

The Role of AcrA in Antibiotic Resistance and Virulence of
***Salmonella enterica* serovar Typhimurium**

By

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Abstract

AcrA is the periplasmic adaptor protein component of the major efflux system AcrB-TolC of *Salmonella* Typhimurium. AcrA of *S. Typhimurium* SL1344 was inactivated and the mutant phenotype characterised. RT-PCR and western blotting were used to confirm expression of *acrB*/AcrB was retained in the *acrA* mutant. The AcrA mutant was hyper-susceptible to a range of antimicrobials and was more susceptible to some agents than strains lacking AcrB and TolC. This is partially explained by the increase in accumulation of Hoechst H33342, a fluorescent substrate of AcrAB-TolC, indicating that the inactivation of *acrA* resulted in reduced efflux activity. Lack of AcrA also attenuated the ability of *S. Typhimurium* to infect INT-407 and RAW 264.7 cells *in vitro* as previously published for AcrB and TolC mutants. The virulence defect of the mutants could not be rescued by addition of supernatant from an infection of INT-407 cells with SL1344 or addition of media conditioned by uninfected INT-407 cells. However, addition of media conditioned by overnight growth of SL1344 was able to ameliorate the virulence defect of the mutants. This suggests that AcrAB-TolC of SL1344 exports a factor/s required for virulence which the mutants are unable to export and that exogenous addition of this factor can restore the virulent phenotype. Inactivation of *acrA* conferred a phenotype distinct to that of inactivation of *acrB* or *tolC* indicating a role for AcrA distinct to that of other protein partners in both efflux of substrates and virulence.

For Jonathan

And My Parents

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Clarification of Contribution to Collaborative work

The following sections or figures were the result of collaborations. Details of the collaboration and the contribution by both parties is clarified below.

Figure 3.11 Transmission Electron Microscopy to examine bacterial structure

Jessica Blair was responsible for setting up and growing the bacterial cultures at the Veterinary Laboratories Agency, Weybridge. The samples were then fixed and electron microscopy images taken by Bill Cooley at the Veterinary Laboratories Agency, Weybridge.

2.11.1 Constructing Green Florescent Protein (GFP) marked strains

The *gfp* encoding pUA66*pacpP* plasmid was a gift from Neil Burton at the University of Birmingham. Jessica Blair was responsible only for transformation of this plasmid into the strains of interest.

Figure 4.4 Confocal microscopy images of INT-407 cells infected with wild-type and efflux mutant strains containing the GFP encoding pUA66*pacpP* plasmid.

Jessica Blair was responsible for the culture of eukaryotic cells, preparing eukaryotic cell infection plates, growing bacterial cultures, infecting the eukaryotic cells and fixing the infections with 1% Formalin. Dr. Rob Shaw (University of Birmingham) stained the eukaryotic cells and took confocal microscopy images.

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List of Abbreviations

Abbreviation	Definition
µg	micrograms
µl	microlitre
µM	micromoles
ABC	ATP-binding cassette
amp	Ampicillin
AMP	Antimicrobial peptide
API	Analytical profile index
<i>aph</i>	Aminoglycoside phosphotransferase
bp	Base pairs
BSAC	British Society for Antimicrobial Chemotherapy
cDNA	Complimentary DNA
CFU	Colony Forming Units
CO ₂	Carbon dioxide
DHPLC	Denaturing High Pressure Liquid Chromatography
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EAEC	Enteroaggregative <i>E. coli</i>
EDTA	Ethylenediaminetetra acetic acid
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
FCS	Foetal calf serum
g	grams
<i>g</i>	gravity
GFP	Green Fluorescent Protein
HBSS	Hanks balanced salt solution
HCL	Hydrochloric acid
INT-407	Human intestinal epithelial cell line
ISO	Iso-sensitest
kan	Kanamycin
kb	Kilobase
L	Litre
LB	Luria Bertani
LEE	Locus enterocyte effacement
LPS	Lipopolysaccharide
M	Molar
MATE	Multidrug and toxic compound extrusion family
MAR	Multiple antibiotic resistance
MDCK	Madin-Darby Canine kidney cells
MDR	Multi-Drug resistant
MEM	Minimal Essential Medium
MIC	Minimum inhibitory Concentration
min	minute
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
mg	milligrams
ml	millilitre

MOE	Multiplicity of Exposure
MOPS	3-(N-morpholino)propanesulfonic acid
mM	millimolar
mRNA	Messenger RNA
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
NCTC	National Collection of typed cultures
NEAA	Non-essential amino acids
ng	nanograms
nm	nanometres
NTS	Non-Typhoidal Salmonella
OD	Optical Density
Omp	Outer membrane protein
PA β N	Phe-arg-beta-naphthylamide
PAMP	Pathogen associated molecular pattern
PAP	Periplasmic adaptor protein
PBP	Penicillin Binding Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RND	Resistance nodulation division
rpm	Revolutions per minute
rRNA	Ribosomal Ribonucleic acid
RAW 264.7	Mouse macrophage cell line
RT-PCR	Reverse-transcriptase PCR
s	Seconds
SCV	Salmonella containing vacuole
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SMR	Small multidrug resistance family
SP	Spacious phagosome
SPI	Salmonella Pathogenicity Island
TBE	Tris-Borate EDTA buffer
TE	Tris-EDTA buffer
TLR	Toll-like receptor
UV	Ultra violet
WT	Wild-type
XLD agar	Xylose Lysine Deoxycholate agar

Chapter One

Introduction

1. Introduction

1.1 *Salmonella*

The genus *Salmonella* is a member of the Enterobacteriaceae family of Gram-negative bacteria. The genus is comprised of two species: *S. enterica* and *S. bongori*. While *S. bongori* mainly infects cold-blooded animals, *S. enterica* is a common cause of infection in warm-blooded animals. *S. enterica* is comprised of six subspecies: *S. enterica* subspecies *enterica* (I), *S. enterica* subspecies *salamae* (II), *S. enterica* subspecies *arizonae* (IIIa), *S. enterica* subspecies *diarizonae* (IIIb), *S. enterica* subspecies *houtenae* (IV), and *S. enterica* subspecies *indica* (VI) and has been classified into more than 2500 serovars according to the Kaufmann-White classification system, based on agglutination reactions with antisera against specific *Salmonella* antigens (Kim *et al.*, 2006). The majority of human and domestic animal infections are caused by only a few serovars including Typhimurium, Enteritidis and Typhi which cause very different illnesses.

1.2 *Salmonella* Infection

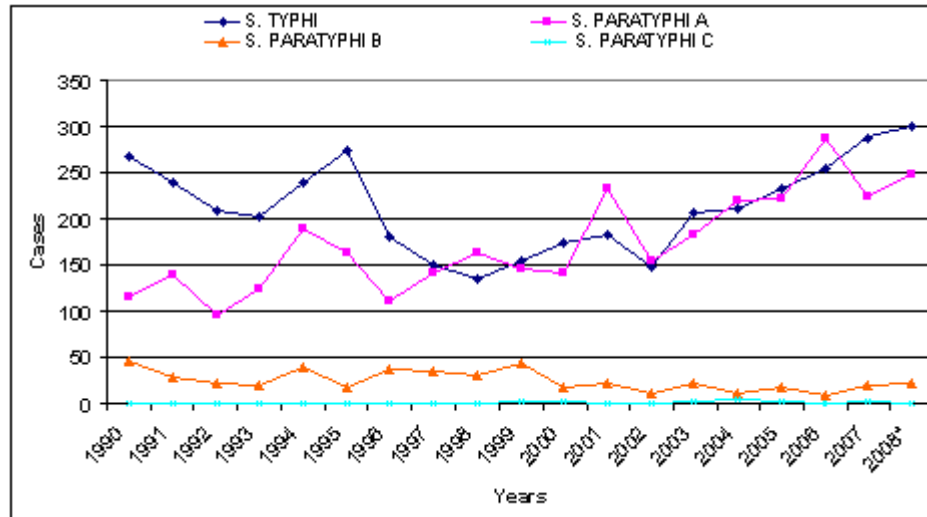
Every year *Salmonella* causes three billion human infections globally (Cooke *et al.*, 2007). Of which, 22 million are caused by the obligate human pathogens *Salmonella* Typhi or Paratyphi which cause invasive systemic infections (Cooke *et al.*, 2007). These result in 200,000 deaths every year, mainly in the developing world (Crump *et al.*, 2004; WHO, 2008). Symptoms include acute fever, diarrhoea or constipation, severe abdominal pain and a maculopapular rash (Ohl and Miller, 2001) and is fatal in 10-15% of cases (Graham, 2002; CDC, 2007). It is significantly less common in the developed world with only 299 reported cases of *S. Typhi*

and 248 cases of *S. Paratyphi A* in England and Wales in 2008, the vast majority of which were contracted by travellers to endemic regions (Figure 1.1 a) (HPA, 2009). The remainder of the cases are caused by non-typhoidal *Salmonella* (NTS) serovars such as *S. Enteritidis* and *S. Typhimurium*, which commonly cause gastroenteritis (Hohmann, 2001; HPA, 2009). Symptoms begin 12-72 hours post infection and include diarrhoea, which can be bloody, abdominal cramps and acute onset fever. Symptoms can last between four to seven days (CDC, 2007) although faecal shedding can continue for up to seven weeks post infection (Hohmann, 2001). Most people recover without treatment as the infection is usually self-limiting but hospitalisation is required in around 20% of cases (Mead *et al.*, 1999). Approximately 5% of patients with gastroenteritis caused by NTS develop bacteraemia, which is a potentially fatal complication that is more common in developing countries (Hohmann, 2001). In tropical Africa *S. Typhimurium* and *S. Enteritidis* are the commonest causes of childhood bacteraemia with a fatality rate of around 30%. Other reported complications of NTS infection include pneumonia, septic arthritis and meningitis (Graham, 2002).

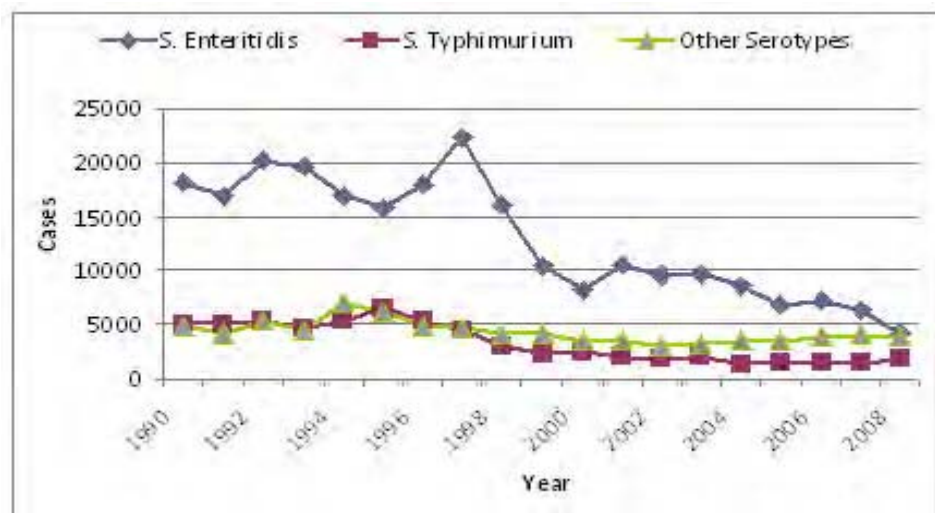
In humans the infectious dose of *S. Typhimurium* has been reported to be between 10^5 - 10^{10} cells (Blaser and Newman, 1982) and there is a negative correlation between the ingested bacterial dose and the median incubation period (Abe *et al.*, 2004). In the developed world 95% of all NTS infections are food borne (Mead *et al.*, 1999) and are often associated with consumption of contaminated poultry or eggs (Cogan and Humphrey, 2003). Other transmission routes include contaminated water, contact with pets (particularly birds and reptiles) and nosocomial transmission (Hohmann, 2001). The susceptibility of individuals to

Figure 1.1 The number of reported *Salmonella* infections in England and Wales (1990-2008)

- a. Number of reported *Salmonella* Typhi, Paratyphi A, Paratyphi B and Paratyphi C infections in England Wales between 1990 - 2008



- b. Number of reported *Salmonella* Enteritidis and Typhimurium infections in England Wales between 1990 – 2008



Data from www.HPA.org.uk

gastrointestinal *Salmonella* infections is dependent on risk factors including the extremes of ages, disruption of commensal gut flora (for example as a result of recent antimicrobial therapy), high gastric pH, malignancy, diabetes, HIV and therapeutic immunosuppression. Complicated infections are far more common in immunocompromised individuals, such as those with HIV or undergoing therapeutic immunosuppression because they lack the effective T-helper-1 (Th1) response necessary to protect against disease caused by intracellular pathogens (Hohmann, 2001; Graham, 2002). Persistent *Salmonella* infections can also occur and individuals are predisposed to this where there is anatomical perturbation (e.g. by kidney stones), abnormalities of the urinary tract or prosthetic devices as these sites can act as foci around which the bacteria can persist (Hohmann, 2001).

In England and Wales there are approximately 20,000 reported cases of NTS every year (HPA, 2007; Rhen *et al.*, 2007) (Figure 1.1 b) and around 200,000 in the USA (Mead *et al.*, 1999) although the actual number of cases is likely to be much higher as the disease is normally self limiting and therefore goes unreported (Rhen *et al.*, 2007). According to the Health Protection Agency (HPA), reported *Salmonella* cases in the UK peaked at 31,482 cases in 1997 but then declined markedly to 9867 reported cases in 2008 (HPA, 2009). This decline was largely due to successful control measures, including wide scale vaccination of chickens, which are a major reservoir of infection (Cogan and Humphrey, 2003). However, since 2005 there has been a rise in the number infections caused by serovars of *Salmonella* against which the vaccine provides no protection (Figure 1.1 b) (HPA, 2009).

In England and Wales the most common *Salmonella* serovars are *S. Enteritidis* and *S. Typhimurium* although the levels of these are dynamic. During the late 1970s *S.*

Typhimurium was the predominant strain. However by the early 1980s *S. Enteritidis* had become more common with 15,153 cases in 1988 and by 1997 *S. Enteritidis* accounted for over 70% of all NTS infections in England and Wales. Provisional data for 2008 suggests that *S. Enteritidis* is still the predominant strain, accounting for 42% of the reported *Salmonella* infections in England and Wales while *S. Typhimurium* accounts for around 18% (Figure 1.1 b) (HPA, 2009). This changing epidemiology is closely linked to infection levels in chickens.

1.3. The *Salmonella* Cell Wall

The evolutionary success of the Gram-negative cell wall is reflected in the abundance of Gram-negative organisms and the wide variety of ecological niches in which they are found. It is responsible for protecting the cell, maintaining cell shape and preventing osmotic lysis. The cell wall of Gram-negative bacteria such as *Salmonella* consists of an outer membrane, above a thin layer of peptidoglycan, and an inner membrane, separated by the periplasm (Beveridge, 1999).

1.3.1 The Inner Membrane

The inner membrane of Gram-negative bacteria is a phospholipid bilayer similar in structure and composition to the eukaryotic plasma membrane (Mühlradt, 1976). Proteins associated with the inner membrane are either integral inner membrane proteins with α -helical membrane spanning domains or lipoproteins bound to the outer leaflet via their N-terminal cysteine residue. The functions of these inner membrane proteins include small molecule transport, protein translocation and lipid biosynthesis (Ruiz *et al.*, 2006).

1.3.2 The Periplasm

The periplasm in *Salmonella* is typically 10-20 nm wide and accounts for approximately 20-40% of the total cell volume (Stock *et al.*, 1977). The periplasm consists of a wide variety of macromolecules including enzymes and chaperone proteins to facilitate protein folding. The periplasmic space also contains a thin layer of peptidoglycan that provides strength and shape to the cell aiding the prevention of osmotic lysis (Ruiz *et al.*, 2006). Peptidoglycan is a polymer of glycan chains crosslinked by oligopeptide molecules producing an elastic matrix (Dmitriev *et al.*, 2005) that is covalently bound to the outer membrane via Braun's lipoprotein (Lpp) (Ruiz *et al.*, 2006).

1.3.3 The Outer Membrane

The outer membrane of Gram-negative organisms is a selectively permeable barrier between the periplasm and the external environment that prevents various toxic compounds entering the cell (Nikaido, 2003; Ruiz *et al.*, 2006). It is an asymmetric lipid bilayer. The inner layer is composed of phospholipids and the outer layer of phospholipids and lipopolysaccharide (LPS) (Doerrler, 2006). LPS is a glycolipid found exclusively in Gram-negative bacteria. *S. Typhimurium* mutants with reduced LPS levels have increased outer membrane permeability and consequently are hyper-susceptible to a range of antibiotics, dyes and detergents as well as suffering leakage of periplasmic enzymes (Vaara, 1993; Ruiz *et al.*, 2006). LPS consists of three parts: the core oligosaccharide, which is attached to the distal oligosaccharide or O-antigen, and the Lipid A domain (Raetz and Whitfield, 2002). The functions of the core oligosaccharide include providing a binding site for the distal polysaccharide side chains and stabilising the outer membrane (Heinrichs *et al.*,

1998). Loss of this core region leads to an increase in the susceptibility of *Salmonella* to many antibiotics (Vaara, 1993). Unlike the core oligosaccharide, which shows very limited structural variation, probably due to its crucial role in membrane stability, the distal O-antigens vary greatly in their length, composition and structure. This antigenic variation is utilised by the Kaufmann-White system to classify *Salmonella* into serovars (Heinrichs *et al.*, 1998; Mimms, 2003). Mutants lacking the O-antigen show greatly reduced virulence in the chick model (Carroll *et al.*, 2004). Lipid A is a highly conserved hydrophobic moiety responsible for anchoring LPS to the outer membrane (Mimms, 2003). It is composed of long fatty acid chains that are linked to a di-glucosamine backbone. Mutations in the Lipid A biosynthetic pathway are often lethal or lead to mutants which are super-susceptible to hydrophobic antibiotics and very large hydrophilic antibiotics which are normally excluded from the cell by the outer membrane (Vaara, 1993). Lipid A is also known as an endotoxin because it is partly responsible for the toxicity of many Gram-negative bacteria, including *Salmonella*, as it activates the mammalian innate immune system via the Toll-like receptor system.

Changes in LPS have been shown to have profound effects on *S. Typhimurium*, including altering the level of protein secretion via type three secretion systems (T3SS). For example, the length of the O-Antigen has recently been linked to the level of secretion of proteins by the type three secretion systems (T3SS) of *S. Typhimurium*. Strains lacking long (containing approximately 35 repeats) and very long (containing approximately 100 repeats) O-Antigens had increased secretion of the T3SS-1 effector protein, SipA, although secretion by T3SS-2 was unaffected (Holzer *et al.*, 2009). In addition, mutation of *waaN*, a gene involved in lipid A

biosynthesis, resulted in reduced secretion of proteins, such as the Sips and Sops, by T3SS-1 of *S. Typhimurium*. There was also a subsequent reduction in pathogenesis in the calf ileal loop model of infection and a loss of ability to invade cultured human epithelial cells (Watson *et al.*, 2000).

Mutations in LPS have also been shown to affect the insertion, folding and stability of membrane proteins. LPS was shown to be required, along with the Skp protein, for folding and insertion of OmpA into phospholipid bilayers. Initially, Skp binds to OmpA, preventing OmpA from folding. This complex then binds to LPS which facilitates insertion and folding into phospholipid bilayers (Bulieris *et al.*, 2003). LPS is also required for the proper folding of PhoE in *E. Coli*. PhoE is an outer membrane porin that is assembled into the outer membrane. Purified LPS with Triton X-100 and divalent cations was able to induce folding of the PhoE protein and the efficiency of folding increased linearly with the amount of LPS (de Cock and Tommassen, 1996). A subsequent study showed that it is the core and lipid A regions of LPS that are required for efficient folding of PhoE (de Cock *et al.*, 1999). A role has also been shown for LPS in the stability of the *S. Typhi* protein OmpC as removal of LPS resulted in a reduction in the thermal stability of the protein (Arockiasamy *et al.*, 2004).

The *S. Typhimurium* outer membrane also contains approximately 60% of the protein found in the entire cell envelope and approximately 90% of the total lipoprotein (Ohl and Miller, 2001). Outer membrane lipoproteins are anchored to the membrane in the same manner as those found on the inner membrane but are found on the inner rather than outer leaflet of the membrane. The integral membrane proteins of the outer membrane are very different from their inner membrane counterparts as they fold to form β -barrel conformations that

span the membrane. These function as channels permitting the influx of nutrients and the export of waste products that would otherwise be unable to traverse the membrane due to its amphipathic nature (Nikaido, 2003; Doerrler, 2006; Ruiz *et al.*, 2006).

1.3.4 Protein Secretion Systems

Multiple membrane associated protein secretion mechanisms have evolved to allow bacteria to secrete proteins across the inner and outer membranes. Many of these systems are required for pathogenicity. Protein secretion mechanisms are categorised into six main groups which are summarised in Table 1.1. 1.4 Pathogenesis and Virulence of *Salmonella* Typhimurium

1.4.1 Colonisation and adhesion

The first stage of *S. Typhimurium* infection is attachment and colonisation of host intestinal tissues. The surface of the host intestine is made up of microvilli separated by crypts and covered with a layer of mucus (Mimms, 2003). The surface is non-uniform as it contains irregularities such as Peyer's patches, which are one to two cm long thickenings of lymphoid tissue in the host ileum (Jepson and Clark, 2001).

After ingestion, infection by *Salmonella* begins by crossing the mucus layer and adhering to the intestinal mucosa. Bacterial attachment is important because it prevents mechanical removal of the bacteria from the intestine. This process is mediated by the plethora of

Table 1.1 Bacterial protein secretion systems

Type	Structure	Mechanism	Function/Features	Examples
I	Three component structure: Inner membrane transporter Periplasmic Adaptor Protein linked to the inner membrane and to the third component, the Outer Membrane channel	Inner membrane transporter uses ATP hydrolysis to power transport of substrates through the outer membrane channel to the extra cellular space.	Transport of a wide variety of proteinaceous and non- proteinaceous substrates to the extracellular space.	Haemolysin A transport system of <i>E. coli</i> , HlyABC (Härtlein <i>et al.</i> , 1983)
II	Consist of approximately 12 components including an outer membrane secretin and a cytoplasmic ATPase (Peabody <i>et al.</i> , 2003; Filloux, 2004; Cianciotto, 2005; Johnson <i>et al.</i> , 2006)	Proteins are transported across the inner membrane by the Sec or Tat pathways before transport across the outer membrane by the type II secretion system.	Transport of a variety of substrates, including some required for virulence, from the periplasm to the extracellular space (Gerlach and Hensel, 2007) Related to the type IV pilus machinery (Peabody <i>et al.</i> , 2003)	Secretion of pullulanase (PulA) by <i>Klebsiella oxytoca</i> (d'Enfert <i>et al.</i> , 1987)
III	Syringe-like mechanism composed of approximately 20 proteins. Spans the inner membrane, periplasm, outer membrane and the membrane of the host cell.	A syringe like mechanisms injects effector proteins directly into the host cell cytoplasm.	Found predominantly in pathogenic bacteria where they are used to deliver effector proteins into the host cell to modulate the host cell behaviour (Ghosh, 2004; Gerlach and Hensel, 2007)	Secretion of Sip and Sop proteins of <i>S. enterica</i> through the Salmonella Pathogenicity Island 1 encoded T3SS (Collazo and Galán, 1997).
IV	The archetypal VirB/D system of <i>Agrobacterium tumefaciens</i> spans the inner and outer membrane and consists of 12 proteins, including an extracellular pilus and three ATPases (Christie <i>et al.</i> , 2005)	Proteins are transferred between cells via a conjugation like mechanism.	Can transport proteins, protein complexes or single stranded DNA either to the extracellular milieu or directly into the host cell cytoplasm. Related to conjugation machinery	Toxin secretion by <i>Bordetella pertussis</i> (Farizo <i>et al.</i> , 2000) VirB/D4 transfer system of <i>A. tumefaciens</i> (Christie, 1997)

V	<p>Autotransporters: A single protein with a signal peptide, passenger domain and translocation unit.</p> <p>Two partner protein system (TPS): Two separate proteins – the transported protein and its transporter</p>	<p>The N-terminal signal peptide directs the protein to the sec pathway through the inner membrane, the passenger domain encodes the effector protein and the C-terminal translocation unit inserts into the membrane forming a β-barrel pore through which the passenger domain is secreted. In autotransporters this is all a single protein in the two partner system the transporter and passenger are two separate proteins (Jacob-Dubuisson <i>et al.</i>, 2004; Gerlach and Hensel, 2007)</p>	<p>Autotransporter proteins mediate their own transport across the outer membrane while TPS proteins require a second protein which acts as a transporter.</p> <p>Secretion of large, virulence related proteins (Jacob-Dubuisson <i>et al.</i>, 2001)</p>	<p>Autotransporter: Pet toxin of <i>E. coli</i> (Eslava <i>et al.</i>, 1998)</p> <p>TPS: Filamentous haemagglutinin secretion through FhaC in <i>B. pertussis</i> (Jacob-Dubuisson <i>et al.</i>, 1999)</p>
VI	<p>Commonly between 15 and 25 proteins but the structure of the system is currently not well defined (Schell <i>et al.</i>, 2007). Include proteins from the cytoplasm, membrane and extracellular space (Bingle <i>et al.</i>, 2008).</p>	<p>Transport is probably driven by ATP hydrolysis. For example by ClpV from <i>S. Typhimurium</i> has ATPase activity (Schlieker <i>et al.</i>, 2005). In the <i>Edwardsiella tarda</i> virulent protein (EVP) T6SS there are 16 proteins. At least 3 are secreted (EvpC, EvpI and EvpP) and 11 are not secreted, of which, one (EvpO) has ATPase activity (Zheng and Leung, 2007).</p>	<p>Export of virulence factors (Schell <i>et al.</i>, 2007)</p> <p>Many components well conserved (Bingle <i>et al.</i>, 2008)</p>	<p>The <i>Edwardsiella tarda</i> virulent protein (EVP) gene cluster has been identified as a T6SS (Zheng and Leung, 2007).</p>

adhesins encoded in the *Salmonella* genome of which an important class are the fimbriae. Fimbriae are filamentous surface structures composed of multiple copies of the fimbrillin subunit and with a small number of copies of an adhesive subunit at the end (Rhen *et al.*, 2007). Type I fimbriae have been linked to adhesion by *S. Typhimurium*. For example, a mutant lacking the *fim* operon could not attach to or invade HeLa cells (Baumler *et al.*, 1996b). This also suggests that adhesion is a pre-requisite for invasion. Different fimbrial adhesins show specificity to different tissue types, for example, the long polar or *lpf* fimbrial operon and the plasmid encoded Pef fimbria mediate adhesion of *S. Typhimurium* specifically to Peyer's patches during murine infection (Baumler *et al.*, 1996a; Baumler *et al.*, 1996b; Baumler *et al.*, 1996c; Ohl and Miller, 2001). Non-fimbrial adhesins include the autotransporter protein MisL. Over-expression of MisL allowed *S. Typhimurium* to bind to fibronectin on coated glass slides and furthermore, increased invasion levels into T84 cells *in vitro* (Dorsey *et al.*, 2005).

1.4.2 Invasion

Salmonella is an intracellular pathogen and it uses multiple mechanisms to cross the intestinal barrier. Firstly, *Salmonella* can be internalised by professional phagocytes. Alternatively, *Salmonella* can invade the intestinal epithelia by inducing its own uptake into non-phagocytic enterocytes, a process called bacterially-mediated endocytosis. This requires a T3SS encoded by a 40 Kb genomic region called *Salmonella* Pathogenicity Island (SPI) 1. Pathogenicity islands are horizontally acquired discrete regions of the genome encoding genes required for pathogenicity. SPI-1 encodes a T3SS which injects bacterial effector proteins into the host cell cytoplasm allowing manipulation of the host cell physiology. These

effector proteins include the Rho-GTPase exchange factors SopE and SopE2, and SopB, an inositol phosphatase, which together activate host rho-GTPases which initiate rearrangement of the host actin cytoskeleton and destabilisation of host cell tight junctions. *S. Typhimurium* mutants lacking any one of these three effectors was still able to invade HeLa cells. However, when *sopE*, *sopE2* and *sopB* were all inactivated the strain was unable to induce actin reorganisation in the host cell, preventing internalisation (Zhou *et al.*, 2001; Haraga *et al.*, 2008). Two further effector proteins SipA and SipC then bind to actin and further modulate its rearrangement to facilitate outward extension of the membrane ruffles and therefore the efficiency of bacterial uptake. SipA promotes polymerisation of actin by reducing the critical concentration required for actin polymerisation and stabilises the actin filaments by inhibiting their depolymerisation (Zhou *et al.*, 2001; Hayward and Koronakis, 2002; McGhie *et al.*, 2009). SipA also works synergistically with SipC which nucleates the actin rearrangement (Hayward and Koronakis, 1999; Hayward and Koronakis, 2002). The host cytoskeleton rearrangement results in prominent membrane protrusions called ruffles which engulf the bacteria leading to their uptake by macropinocytosis (Haraga *et al.*, 2008).

The actin cytoskeleton rearrangements which cause ruffles are only transient and after bacterial uptake normal host cell architecture is restored; a process which is also bacterially mediated (Fu and Galan, 1999; Groisman and Mouslim, 2000). The SptP protein is injected by the SPI-1 encoded T3SS after secretion of the ruffle promoting proteins such as SopE. It is a GTPase activating protein that is directly responsible for the reversal of the cytoskeletal rearrangements, possibly to subvert host immune responses and to maintain viability of the host cell (Fu and Galan, 1999).

Another way *Salmonella* become internalised is via membranous epithelial (M) cells. M cells are specialised epithelial cells which form the follicle-associated epithelium (FAE) overlying the Peyer's patches. These cells capture sample antigens from the intestine by pinocytosis, a non-specific process. The antigens are then transported to the lymphoid tissue in the Peyer's patches below, where a protective immune response is initiated (Jepson and Clark, 2001; Haraga *et al.*, 2008). However, the ability of M-cells to non-specifically transport a wide variety of substrates leaves them vulnerable to exploitation by *Salmonella* (Jepson and Clark, 2001). *S. Typhimurium* has been shown to preferentially adhere to M-cells in orally infected mice with 34 fold more bacteria found per unit area of M-cells than per unit area of other enterocyte cells demonstrating the importance, and the preference of *Salmonella*, for this route of invasion (Clark *et al.*, 1994; Ohl and Miller, 2001). Invasion of M-cells by *Salmonella* causes damage resulting in sloughing of the FAE in both the mouse and calf ileal loop models (Jones BD, 1994; Penheiter *et al.*, 1997), allowing the bacteria to disseminate into adjacent sub-epithelial tissues (Jepson and Clark, 2001).

Finally, it was observed that while *Salmonella* lacking functional SPI-1 could cause a lethal infection in mice after oral administration, and reach the spleen (Galan and Curtiss, 1989), it was not doing so via M cells or Peyer's patches (Penheiter *et al.*, 1997). Vazquez-Torres and colleagues reported *Salmonella* could also be taken up from the gastrointestinal system, by tissue macrophages expressing the CD18 antigen, and transferred directly into the bloodstream. Furthermore, *Salmonella* were unable to disseminate to the liver and spleen in CD18 deficient mice during oral challenge but not during peritoneal challenge (Vazquez-

Torres *et al.*, 1999). It has been suggested that this could be significant to the development of systemic immunity (Vazquez-Torres *et al.*, 1999).

1.4.3 Intra-cellular phase of infection

Once *Salmonella* have gained access to the sub-epithelial tissue they encounter many other cell types including macrophages which engulf the bacterial cells. The ability of *Salmonella* to survive within the macrophage is an important virulence determinant as mutants defective for macrophage survival in an *in vitro* assay were shown to be avirulent in the mouse after intraperitoneal infection (Fields *et al.*, 1986).

Once inside the macrophages the *Salmonella* must evade the antimicrobial function of the phagocytes and replicate in the hostile intracellular environment. Immediately after uptake the *Salmonella* reside in a spacious phagosome (SP) formed by closure of the membrane ruffle (Alpuche-Aranda *et al.*, 1994). This SP then shrinks to form a *Salmonella* containing vacuole (SCV). The SCV transiently displays markers of the early endocytic pathway but does not follow the normal phagolysosomal cycle as *Salmonella* interferes with this process by delaying or preventing lysosomal fusion and therefore acidification of the phagosome (Alpuche Aranda *et al.*, 1992; Garvis *et al.*, 2001; Steele-Mortimer, 2008). However, after a lag phase the SCV does become slightly acidic (pH 5-6). This pH change is not bacteriocidal to the *Salmonella* but acts as an important signal for the activation of genes in SPI-2 which are required for intramacrophage survival and therefore suggests that the change in pH is critical to the virulence of *Salmonella* (Alpuche Aranda *et al.*, 1992; Rathman *et al.*, 1996; Cirillo *et al.*, 1998).

The pH change is detected by the two component system PhoP/PhoQ (Bearson *et al.*, 1998). This system detects environmental signals and in response controls expression of over 200 virulence related genes including those encoded by SPI-2 (Miller and Mekalanos, 1990; Lucas and Lee, 2000). It consists of an inner membrane sensor kinase, PhoQ, and a transcriptional activator, PhoP. The sensor component, PhoQ, detects levels of extracellular cations via two non-interacting binding sites near its C-terminus (Vescovi *et al.*, 1997). Low pH levels cause PhoQ to promote the phosphorylation of PhoP. This causes conformational changes in the DNA binding domain of PhoP, allowing it to bind to target promoter sequences and promote transcription of PhoP regulated genes (Yamamoto *et al.*, 2002; Shin and Groisman, 2005). The absence of PhoPQ caused attenuation of virulence in the mouse model of infection after intraperitoneal infection (Miller *et al.*, 1989). This system is also critical in directing adaptation to Mg^{2+} limiting conditions and providing resistance to many antimicrobial peptides including mammalian defensins (Miller *et al.*, 1989; Bearson *et al.*, 1998; Groisman, 2001).

To survive within the phagosome *Salmonella* must endure the bactericidal environment containing lysosomal hydrolases and many antimicrobial peptides, reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) (Knodler and Steele-Mortimer, 2003; Haraga *et al.*, 2008). The ability to survive within this environment is an important virulence determinant. Microarray studies have shown differential regulation of over 900 *Salmonella* genes upon phagocytosis by murine J774-A cells *in vitro* showing the scale of response required to the change in environment (Eriksson *et al.*, 2003). Pathogen associated molecular patterns (PAMPs) recognised by the host as an indicator of infection

are down-regulated or altered to avoid detection. For example SPI-1 encoded flagellin, which is recognised by Toll-like receptor (TLR) 5, is dramatically down-regulated in response to PhoP/Q signalling (Miao *et al.*, 2006). Significant LPS remodelling also occurs in response to PhoP/Q signalling to prevent detection by TLR-4 (Guo *et al.*, 1997).

SPI-2 encodes a T3SS that is required for intracellular survival and is expressed by intracellular *Salmonella* (Ochman *et al.*, 1996; Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Eriksson *et al.*, 2003; Hautefort *et al.*, 2008). Conversely, expression of SPI-1 which is required for invasion, is constantly suppressed when inside macrophages and is only expressed during the late stages of infection of epithelial cells *in vitro* (Eriksson *et al.*, 2003; Hautefort *et al.*, 2008). *Salmonella* lacking *spiA*, a component of SPI-2, had a reduced capacity to survive within macrophages and could not kill BALB/c mice after intraperitoneal infection (Ochman *et al.*, 1996). The T3SS machinery translocates effector proteins across the vacuolar membrane into the host cell cytosol allowing the bacteria to modulate its external environment. The effectors participate in various SPI-2 functions including inhibition of lysosomal fusion to the phagosome (Uchiya *et al.*, 1999), *Salmonella* induced filament (Sif) formation, maintenance of the vacuolar membrane integrity (Beuzón *et al.*, 2000) and avoiding the damaging effects of ROIs and RNIs by inhibiting production of NADPH and iNOS as well as preventing the free radicals reaching the SCV (Vazquez-Torres *et al.*, 2000; Gallois *et al.*, 2001; Chakravorty *et al.*, 2002; Knodler and Steele-Mortimer, 2003; Waterman and Holden, 2003).

Approximately 4-6 hours after infection *Salmonella* begin to replicate within the SCV and this is accompanied by the production of *Salmonella*-induced filaments (Sifs). These are

membranous filaments that project outwards from the SCV into the cell along the microtubule network. They are formed following fusion of the SCV with late endocytic compartments and have a membrane composition similar to that of the SCV (Brumell *et al.*, 2001; Birmingham *et al.*, 2005). The function of Sifs is not entirely understood however they cause elongation of the vacuole which could improve nutrient availability, allowing for the rapid bacterial replication which occurs simultaneously (Birmingham *et al.*, 2005; Haraga *et al.*, 2008).

Salmonella can utilise the mobility of the macrophage to aid dissemination of infection via the host lymphatic system while evading the host immune response. Worley and colleagues have shown that *Salmonella* can enhance the motility of the infected macrophages via the protein SrfH which is secreted by the SPI-2 T3SS. While inside macrophages the expression of this gene increases by almost 100-fold. The SrfH protein is secreted across the membrane of the *Salmonella* containing vacuole (SCV) and interacts with a host regulator of motility, TRIP6, promoting the motility of the macrophage. In orally infected mice, this speeds up the systemic spread of infected cells away from the intestine and into the blood stream as well as other host tissues (Worley *et al.*, 2006).

1.4.4 Dissemination of infection

In the early stages of infection *Salmonella* growth is exponential and in the murine model of salmonellosis this stage sees the spread of bacteria from the blood to the spleen and liver (Grant *et al.*, 2008). Further dissemination of infection depends on the bacteria escaping the original cell and moving on to infect other cells. This occurs when the host cell bursts, releasing the intracellular bacteria. Host cell lysis is a stochastic process which is

independent of the number of bacteria infecting the cell (Brown *et al.*, 2006; Mastroeni *et al.*, 2009). Bacteria are only extracellular for a very short time as opsonisation by complement factors occurs rapidly leading to phagocytosis of the *Salmonella*. Due to the massive excess of uninfected host cells compared to the number of bacteria, continuous spread of the bacteria to new host cells occurs (Brown *et al.*, 2006).

The number of bacteria in each infected host cell is described by a skewed distribution where most infected cells contain a small number of bacteria while only a few contain higher bacterial numbers (Sheppard *et al.*, 2003; Brown *et al.*, 2006; Mastroeni *et al.*, 2009). Through the course of murine infection there is a simultaneous increase in the total bacterial number in the tissue and the number of infected cells while the distribution of bacteria per infected host cell remains fairly constant (Sheppard *et al.*, 2003). At the same time there is an increase in the number of discrete infection foci within the tissue. These are spatially separated sites of infection founded by a singular bacterium that expands clonally and can form multi-cellular pathological lesions surrounded by healthy tissue. The foci are functionally independent and spread of bacteria between foci is not seen (Sheppard *et al.*, 2003; Mastroeni *et al.*, 2009). In summary, progression of *Salmonella* infection *in vivo* is predominantly due to an increase in the number of infected cells and the foundation of new infection foci and not by replication of large numbers of *Salmonella* within the same phagocyte.

1.4.5 Model systems to study *Salmonella* infection

Animal models of *Salmonella* infection are commonly used to study mechanisms of pathogenesis and virulence relevant to both human and animal infections. However, careful

thought should be given to the appropriate model as different *Salmonella* serovars, infection models and inoculation route can produce very different symptoms and pathology and therefore could affect conclusions drawn from experiments.

One of the most common animal models for *Salmonella* infection is the mouse and this model has been instrumental in the identification of multiple virulence mechanisms of *S. Typhimurium* (Galan and Curtiss, 1989; Hensel *et al.*, 1995). However, a major limitation of this model for studying NTS is that oral infection with *S. Typhimurium* causes a systemic infection, similar to typhoid fever, which is very different from the gastrointestinal symptoms that characterise human infection. Four to eight days after oral infection with *S. Typhimurium* mice develop an elevated temperature but do not develop diarrhoeal symptoms and exhibit a very different intestinal pathology (Kraus *et al.*, 1999; Santos *et al.*, 2001). Murine *S. Typhimurium* infections culminate in a systemic disease with rapid multiplication of bacteria in the spleen and liver (Santos *et al.*, 2001). However, treatment of mice with streptomycin before oral infection with *S. Typhimurium* results in diarrhoea and colitis with very similar pathology to that seen in humans making this model more useful for the study of *S. Typhimurium* pathogenesis in humans (Barthel *et al.*, 2003). The mouse model of infection has also been inoculated via intra-peritoneal injection. This is useful for studying some aspects of virulence and pathogenicity relevant to systemic infection but is not informative about earlier stages and challenges of infection, such as colonisation and bile resistance, which *Salmonella* have to overcome to cause infection via the oral inoculation route.

An alternative model for the study of *S. Typhimurium* infection is Calves. In contrast to murine infections, bovine oral infections with *S. Typhimurium* result in an enteric disease characterised by diarrhoea and similar pathology to human infections making it a more useful model for the study of pathogenesis in humans. However, other serotypes of *Salmonella* cause different disease syndromes. For example, cattle infected with *Salmonella* Dublin, a serovar known to be associated with infection of cows, can result in abortion and a prolonged carriage state which is not characteristic of *S. Typhimurium* infections (Wray and Sojka, 1981; Santos *et al.*, 2001).

Calf ligated ileal loops can be used to model the early stages of infection and have been used to characterise some virulence factors such as member of SPI1 (Clarke and Gyles, 1987; Frost *et al.*, 1997; Zhang *et al.*, 2002). In this model the intestine of anaesthetised calves is ligated to form multiple small loops, which are each separately infected with *S. Typhimurium* and the course of infection and resulting pathology can be sampled at different time points post infection. A major limitation of this model is that infection can only be studied for approximately 12 hours but a further refinement of the technique by Coombes and colleagues (2005) which involves unblocking the small intestine after infection allows the animals to survive for up to five days post operation (Santos *et al.*, 2001; Coombes *et al.*, 2005). Major benefits of this improved system include the extended length of infection that can be studied and the ability to study an infection of intestinal tissue linked to the blood supply and lymphatic system.

In addition to the vertebrate models of *Salmonella* infection that have been discussed, invertebrate models, such as the nematode *Caenorhabditis elegans*, have also been used. *S.*

S. Typhimurium SL1344 is able to establish a persistent infection and proliferate within the *C. Elegans* intestine and the infection is lethal within approximately 5 days (Aballay *et al.*, 2000). Mutants that had attenuated virulence in other animal models also showed a reduced ability to kill the worm showing that many of the molecular mechanisms required for infection of humans and animals are also required for infection of the worm (Aballay *et al.*, 2000; Labrousse *et al.*, 2000; Tenor *et al.*, 2004).

While animal experiments are invaluable in the study of infection and host pathogen interactions, the experiments can be time consuming as well as expensive. A very common alternative is infection of cultured mammalian cells *in vitro*. This approach has been invaluable in the elucidation of the molecular mechanisms of host cell invasion by *Salmonella* such as the T3SS encoded by SPI-1 (Galan and Curtiss, 1989; Darwin and Miller, 1999). A major advantage of this system is that it allows the interaction between a pathogen and a single type of host cell to be studied. This reductionist approach removes much of the complexity of studying infection in a whole animal. In addition, the interaction between *Salmonella* and human cells can be studied. Common cell lines used to study *S. Typhimurium* infection include HeLa, CaCo2, Hep2 and INT-407 (Dibb-Fuller *et al.*, 1999; Hurley and McCormick, 2003). There are obvious drawbacks to studying a complex host pathogen interaction with only a single cell type. However, tissue culture experiments aid the formation of hypotheses about animal and human infections which can then be tested in the appropriate animal model in a hypothesis driven manner to substantiate any findings.

1.5 Treatment of *Salmonella* Infections

There is no single, routine antimicrobial therapy recommended to treat otherwise healthy individuals displaying mild to moderate symptoms of gastrointestinal infection (Hohmann, 2001). Treatment of these individuals with antimicrobial therapy remains controversial because it is often not possible to reliably identify the causative agent of a gastrointestinal infection upon clinical presentation and inappropriate prescribing can result. Furthermore, the antibiotic treatment of patients with NTS can actually prolong, rather than reduce, the period of faecal shedding and so clinical relapse may be more common in the treated group (Nelson *et al.*, 1980; Hohmann, 2001). This relapse is probably a result of perturbation of commensal gut flora by the antimicrobial therapy, leading to a reduction in its protective effects. However, treatment is always recommended for patients who are severely ill, at the extremes of age or who are for other reasons deemed to be at risk of more complicated or disseminated infection, but only after the causative agent has been confirmed by isolation from blood and/or faecal samples. When a course of antimicrobial therapy is required the drugs of choice include fluoroquinolones (DNA gyrase inhibitors), such as ciprofloxacin, or trimethoprim (a dihydrofolate reductase inhibitor). Trimethoprim can also be administered in combination with a member of the sulphonamides, usually sulphamethoxazole (a competitive inhibitor of enzymes required for nucleic acid synthesis). This combination is known as co-trimoxazole and is used because of the synergistic action of the component parts but is only used as a last resort during invasive infection due to its association with rare but severe side effects (BNF, 2007).

1.6 Antibiotic Resistance

Since the introduction of penicillin in 1944, the use of therapeutic antibiotics has become commonplace and it is estimated that about 100,000 tons of antibiotics are produced annually worldwide (Nikaido, 2009). As predicted by Fleming in his nobel prize speech in 1945 (Fleming, 1945), the emergence of antibiotic resistance has been an inevitable consequence. Multiple studies have shown that the overuse and misuse of antibiotics to treat human infections has contributed to the growing problem of antibiotic resistance (Cohen, 2000). For example, a study by the European Antimicrobial Resistance Surveillance System (EARSS) showed that an increase in penicillin resistance in *Streptococcus pneumoniae* isolates in Europe between 1998 and 1999 correlated with an increase in use of beta-lactam and macrolide antibiotics (Bronzwaer *et al.*, 2002). Another example is a study which linked an increase in the prescription of fluoroquinolone antibiotics for urinary tract infections in the Netherlands to an increase in isolation of resistant *E. Coli* (Goettsch *et al.*, 2000).

Another factor in the emergence of antibiotic resistance is the use of antibiotics in veterinary medicine and the use of prophylactic antibiotics in animal feed to prevent infection and promote growth in livestock. This has contributed to the emergence of resistance in bacteria which are also pathogenic to man. This presents a potential public health risk from zoonotic infections caused by resistant bacteria including *Salmonella* and *Campylobacter* which are often food borne. A major reason for this emerging resistance is that many drugs used to treat animals are also used in human treatment or are structurally very similar to drugs used to treat humans. For example, the fluoroquinolone enrofloxacin, which has been widely used to treat infections in animals, will select mutants *in vitro* resistant to both nalidixic acid

and ciprofloxacin, which are both fluoroquinolones used in human treatment (Giraud *et al.*, 1999). The implication of this is that the consumption of enrofloxacin resistant zoonotic pathogens such as *Salmonella*, could cause infection in humans that is resistant to ciprofloxacin, a clinically useful antibiotic (McEwen and Fedorka Cray, 2002; Piddock, 2002; Tornroth-Horsefield *et al.*, 2007; BNF, 2009).

Antibiotic resistance is either chromosomally encoded or acquired through transferable elements. Chromosomally mediated resistance occurs when mutations conferring resistance are fixed in the population when selection pressure is applied due to exposure to a particular antibiotic or class of antibiotics. The number of resistant bacteria then increases as the resistant strain spreads from animal to animal or infects humans (Piddock, 2002). Alternatively antibiotic resistance can be transferable. This type of resistance was first recognised when plasmid encoded resistance genes from *Shigella* transferred to *E. coli*. Another well studied example is the transfer of the *mecA* gene, which confers methicillin resistance, to *Staphylococcus aureus*. The gene encodes a novel penicillin binding protein, PBP2A, which can now be isolated from up to 90% of all antibiotic resistant clinical isolates (Walsh, 2003). Transferable resistance is easily spread between strains and between species.

Resistance of *Salmonella* to older antibiotics such as chloramphenicol and ampicillin have been present for many years and treatment failures have been reported from strains which are resistant to ciprofloxacin (Piddock *et al.*, 1990; Wain *et al.*, 1997; Parry, 2003; Parry and Threlfall, 2008). *Salmonella* isolates are commonly resistant to more than one antibiotic. Multiple drug resistance (MDR) are defined as resistance to antibiotics from three or more classes. MDR *Salmonella* is commonly resistant to ampicillin, chloramphenicol, streptomycin,

sulphonamides and tetracycline. This phenotype is associated with *S. Typhimurium* DT104 and emerged in cattle in the early 1990s and is often abbreviated to ACSSuT (Randall *et al.*, 2004).

1.6.1 Mechanisms of Antibiotic Resistance

There are four major mechanisms which mediate antibiotic resistance (i) inactivation of the antibiotic, (ii) modification of the target molecule, (iii) metabolic bypass and (iv) efflux of the antibiotic. Many bacteria commonly inactivate antibiotics by producing enzymes which modify the chemically reactive site of the antibiotic molecule causing it to lose its antimicrobial activity. A very widespread example of this type of resistance is the destruction of β -lactam antibiotics by β -lactamase producing bacteria. β -lactamases hydrolyse the reactive β -lactam ring in many clinically important antibiotics, such as penicillins and cephalosporins, negating their antimicrobial activity (Hawkey, 1998). For example the AmpC β -lactamase enzymes provide resistance to the cephalosporins in multiple bacterial species including *Salmonella enterica*, *E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Enterobacter spp.* (Winokur *et al.*, 2000; Jacoby, 2009).

Antibiotic resistance in pathogenic bacteria is also achieved by modifying the target molecule of the antibiotic, rendering it insensitive to the drug, while still retaining its function within the organism. An example of this is quinolone resistance in *Salmonella* and *E. coli* caused by mutations in the *gyrA* gene that encodes the A subunit of the DNA gyrase enzyme. DNA gyrase is an essential enzyme involved in DNA supercoiling. In resistant clinical isolates mutations are most commonly detected close to the DNA binding region of the enzyme, in the Quinolone Resistance Determining Region (QRDR) and more specifically

mutations are most common at serine 83 and aspartate 87 (Griggs *et al.*, 1996; Piddock, 2002). Mutations in this region reduce binding affinity of quinolones to the gyrase enzyme and therefore decrease susceptibility (Willmott and Maxwell, 1993; Hopkins *et al.*, 2005). Another example of alteration of the target molecule is in resistance to rifampicin (Spratt, 1994). Rifampicin exerts its antimicrobial effect by binding to the β subunit of RNA polymerase at a site separate from the active site. In *E. coli* and *Mycobacterium tuberculosis* resistance to rifampicin occurs due to mutations in the *rpoB* gene which encodes the β -subunit of the RNA polymerase (Jin and Gross, 1988; Telenti *et al.*, 1993). This leads to resistance because rifampicin cannot bind to the mutated RNA polymerase. A further example is low level mupirocin resistance in *S. aureus*. Mupirocin works by irreversibly binding to the isoleucyl tRNA synthetase and therefore preventing protein synthesis. In isolates of *S. aureus* with low level mupirocin resistance (MIC of 8 to 256 $\mu\text{g/ml}$) valine to phenylalanine mutations were found in the isoleucyl tRNA synthetase protein, IleS, at residue positions 588 and 631 (Antonio *et al.*, 2002).

An additional mechanism by which bacteria become resistant to antibiotics is by metabolic bypass. This means that the inhibited reaction is circumvented for example by recruiting novel enzymes to bypass the metabolic pathways that are inhibited by antibiotic treatment. Probably the best characterised example of resistance by metabolic bypass is the acquisition of, PBP2a, an alternative Penicillin Binding Protein (PBP) in methicillin resistant *S. aureus* (MRSA) (Hawkey, 1998). This novel enzyme is encoded by the *mecA* gene. The PBP2a enzyme is produced in addition to the other PBPs of *S. aureus* but PBP2a is not inhibited by antibiotics such as methicillin or other β -lactams. It is therefore still able to fulfil its role as a

transpeptidase in crosslinking peptidoglycan precursors so that a normal cell wall is produced even in the presence of otherwise lethal β -lactam concentrations (Inglis *et al.*, 1988; Tesch *et al.*, 1988). Another example of metabolic bypass is high level mupirocin resistance in *S. aureus*. The majority of isolates with high level resistance to mupirocin (MIC of ≥ 1000 $\mu\text{g/ml}$) have acquired a novel isoleucyl RNA synthetase, encoded by the *mupA* gene, which fulfils the function of the chromosomally encoded IleS but is not inhibited by mupirocin (Hodgson *et al.*, 1994; Udo *et al.*, 2001; Patel *et al.*, 2009). Furthermore, sulphonamide antibiotics work by competitively inhibiting the enzyme dihydropteroate synthetase. Resistance occurs by acquisition of a plasmid encoded enzyme which performs the same function but is resistant to far higher levels of sulphonamide (Wise and Abou-Donia, 1975).

1.6.2 Efflux as a Mechanism of Resistance

The fourth major mechanism of resistance is active efflux of antibiotics. Antimicrobial compounds are actively pumped out of the cell through membrane spanning efflux pumps, keeping the internal concentration below levels that are toxic to the bacteria. This mechanism is responsible for the intrinsic resistance that many Gram-negative bacteria have to certain classes of antibiotic and confers resistance to multiple classes of antibiotics, dyes and detergents which are structurally distinct (Li and Nikaido, 2004; Piddock, 2006a). Increased resistance via efflux can be due to either increased expression of the efflux pump, often due to perturbations of the regulatory network, or mutation making efflux more efficient (Piddock, 2006a).

Antibiotic resistance due to over-expression of efflux pumps is a growing clinical problem in multiple bacterial species. For example, over-expression of the OprM efflux pump protein was detected in 11 patients with MDR *P. aeruginosa* infections (Ziha-Zarifi *et al.*, 1999) while the *mexB*, *mexD*, *mexF* or *mexY* genes were over-expressed in fluoroquinolone resistant isolates (Oh *et al.*, 2003). Multidrug resistant isolates of *Campylobacter jejuni* from both humans and poultry were shown to over-express *cmeB* or *cmeF* (Pumbwe *et al.*, 2004) and over-expression of *acrAB*/AcrAB in *Salmonella* and *E. coli* has been implicated many times as a resistance mechanism in both human clinical and veterinary isolates (Piddock *et al.*, 2000; Webber and Piddock, 2003; Baucheron *et al.*, 2004b; Chen *et al.*, 2007). MDR bacteria can also over-produce more than one efflux system simultaneously. For instance, of 450 clinical isolates of ticarcillin resistant *P. aeruginosa* 46% over-expressed *mexAB-oprM* and 58% over-expressed *mexXY*; while 28% of isolates simultaneously over-expressed both systems (Hocquet *et al.*, 2007). Enhanced efflux is also implicated in rapidly emerging clinical resistance to new antimicrobials such as tigecycline, for which over-expression of *acrAB* was detected in *E. coli* and *Enterobacter cloacae* (Keeney *et al.*, 2007; Keeney *et al.*, 2008).

The plethora of efflux pumps which have been associated with MDR have been classified into the following five families: the ATP binding cassette (ABC) superfamily; the major facilitator superfamily (MFS); the multidrug and toxic compound extrusion (MATE) family; the small multidrug resistance (SMR) family; and the resistance nodulation division (RND) superfamily. This classification is based on the number of components from which the pumps are composed, the energy source on which they run, the class of substrate they

transport and the number of membrane spanning regions (Putman *et al.*, 2000; Piddock, 2006a).

1.7 The RND Superfamily of Efflux Pumps

Members of the RND protein family are permeases with 12 α -helical transmembrane domains and two large extra-cytoplasmic hydrophilic loops (Saier *et al.*, 1994; Saier *et al.*, 1998; Tseng *et al.*, 1999). This unusual protein structure is a result of an internal gene duplication event before divergence of the family members (Dinh *et al.*, 1994; Saier *et al.*, 1994). Members of the RND superfamily of efflux pumps are found ubiquitously throughout the Bacteria, Archaea and Eukaryotes and are divided phylogenetically into seven families (Tseng *et al.*, 1999; Saier and Paulsen, 2001). Families one to three are found in the Gram-negative bacteria, family four is found in both Gram-positive and Gram-negative bacteria, family five are restricted to the Gram-positive bacteria, family six is found in the eukaryotes and family seven is found in the archaea and spirochetes (Tseng *et al.*, 1999; Saier and Paulsen, 2001).

The three families found in the Gram-negative bacteria function in complex with two other proteins, an outer membrane channel and a periplasmic adaptor protein (previously known as membrane fusion protein), to form a tri-partite efflux pump that spans both the inner and outer membrane. The RND pump is situated in the inner membrane and utilises the proton motive force to efflux a wide variety of substrates across the entire cell envelope to the extracellular space without the creation of any periplasmic intermediates (Tikhonova and Zgurskaya, 2004). These multi-protein complexes can transport a wide variety of substrates and their phylogeny correlates with substrate specificity of the pump (Saier *et al.*, 1998;

Saier and Paulsen, 2001). Family one exports heavy metals, family two transport many structurally diverse drugs and family three export lipo-oligosaccharides involved in nitrogen fixation during nodulation (Saier *et al.*, 1998; Saier and Paulsen, 2001). The phylogenetic relationship of the RND pump correlates well with that of the periplasmic adaptor protein (PAP) component suggesting that the two components co-evolved, however this is not true of the outer membrane channels which appear to have evolved independently (Paulsen *et al.*, 1997; Saier and Paulsen, 2001).

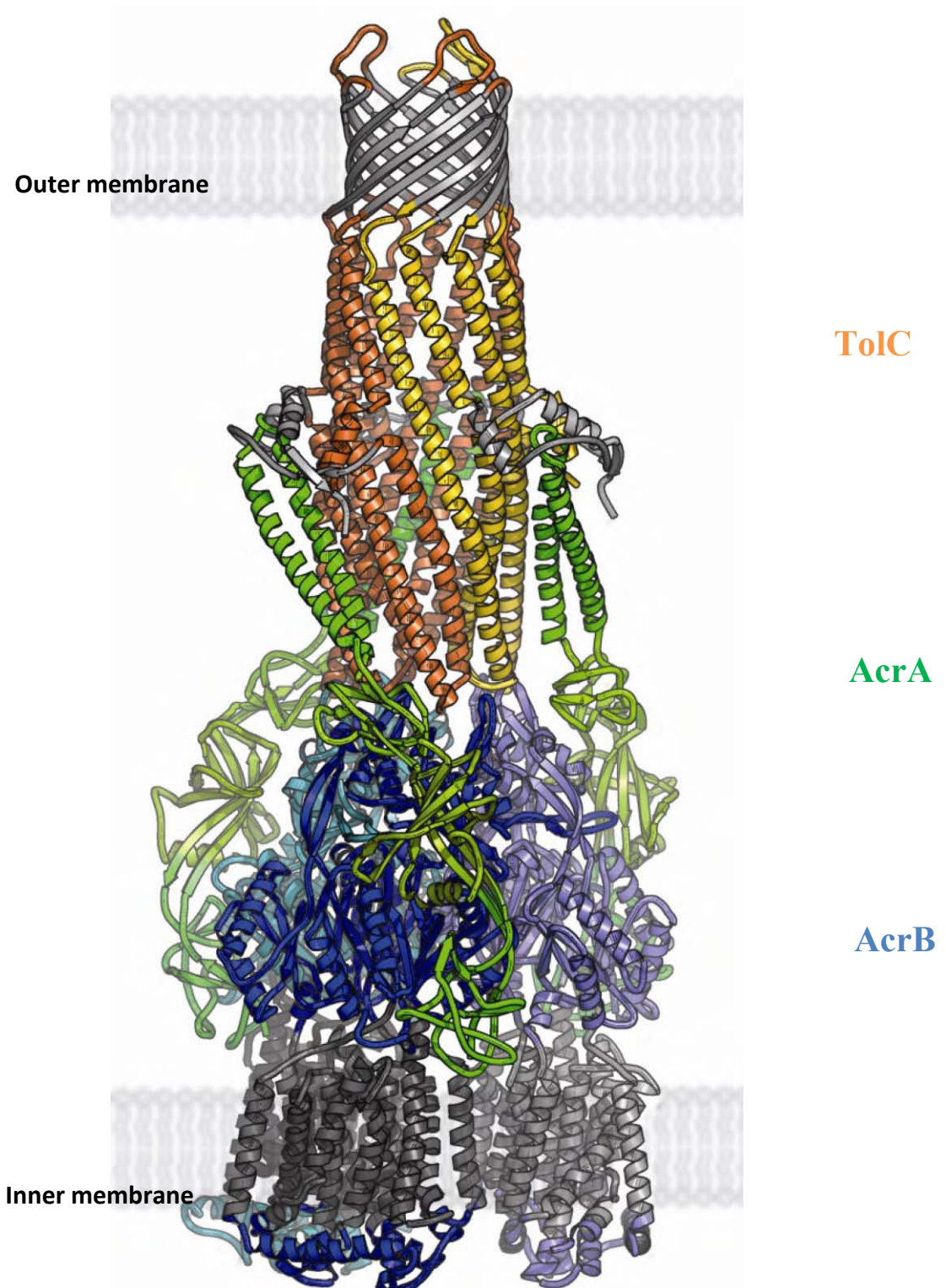
The periplasmic adaptor proteins were previously known as membrane fusion proteins. They were so called due to significant sequence homology to the membrane fusion protein of simian paramyxovirus 5 (Dinh *et al.*, 1994). However, the term membrane fusion protein is misleading and does not reflect the function of this family of proteins. The term periplasmic adaptor protein (PAP) is becoming more common and has been adopted throughout this thesis.

Five RND type efflux pumps (along with corresponding PAP) have been detected in *S. Typhimurium*: AcrAB, AcrEF, MdtABC, MdsABC and AcrD which is not encoded alongside its own periplasmic adaptor protein.

1.8 The AcrAB-TolC Efflux Pump

The three components of AcrAB-TolC efflux pump are AcrB, the inner membrane RND transporter protein, AcrA, the periplasmic adaptor protein (PAP) and TolC, the outer membrane protein channel (Figure 1.2 and 1.3). Its substrates are structurally diverse with different charges and a range of molecular weights. They include antibiotics from multiple

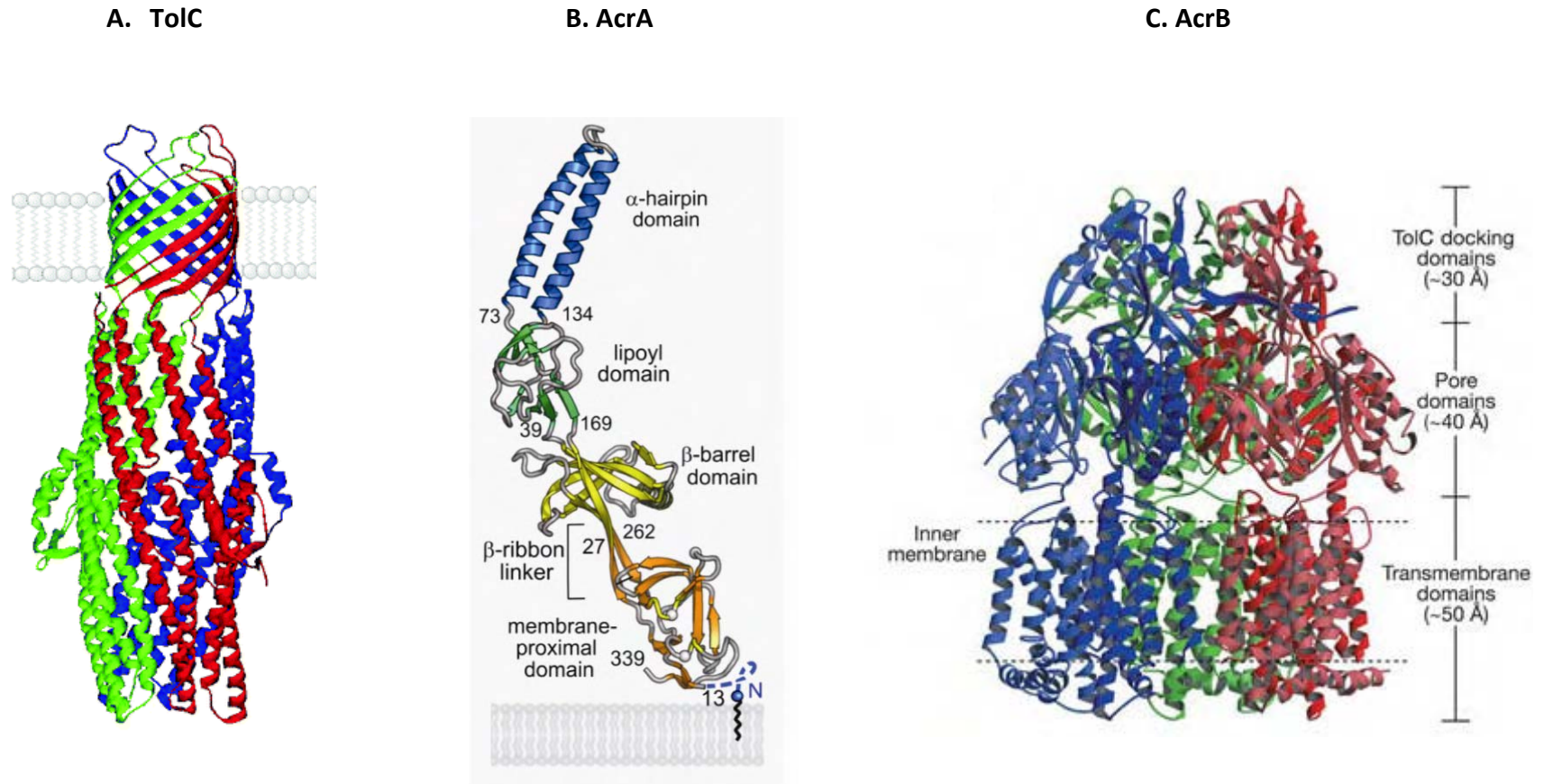
Figure 1.2 The assembled structure of the AcrAB-TolC tri-partite efflux system



From Blair and Piddock, 2009

Image originally provided by Martyn Symmons, University of Cambridge.

Figure 1.3 The crystal structures of the components of the AcrAB-TolC efflux pump of *E. coli*



From Koronakis *et al.*, 2004

Personal communication Martyn
Symmons (University of Cambridge)

From Murakami *et al.*, 2002

classes, including the fluoroquinolones (e.g. ciprofloxacin), β -lactams (e.g. oxacillin) and the tetracyclines (e.g. tetracycline), detergents including SDS, Triton X-100 and bile salts, as well as various dyes and disinfectants (Yu *et al.*, 2003; Eaves *et al.*, 2004b; Poole, 2005; Piddock, 2006a; Seeger *et al.*, 2008a; Tatsumi and Wachi, 2008).

AcrAB-TolC and other members of the RND family, have been associated with intrinsic resistance to a range of antibiotics, dyes, disinfectants and detergents in various members of the Gram-negative bacteria (Okusu *et al.*, 1996; Piddock *et al.*, 2000; Nikaido and Zgurskaya, 2001; Baucheron *et al.*, 2002; Eaves *et al.*, 2004b; Baucheron *et al.*, 2005; Buckley *et al.*, 2006; Nishino *et al.*, 2006). The AcrAB-TolC system is also implicated in the development of clinically relevant levels of resistance to various antimicrobial agents including the fluoroquinolones in both *S. enterica* and *E. coli* (Oethinger *et al.*, 2000; Piddock *et al.*, 2000; de Cristobal *et al.*, 2006; Webber *et al.*, 2008). Over-expression of components of AcrAB-TolC has been associated with antibiotic resistance in both clinical and veterinary isolates of *S. enterica* as well as in laboratory mutants (Giraud *et al.*, 2000; Piddock *et al.*, 2000; Wang *et al.*, 2001; Baucheron *et al.*, 2002; Webber and Piddock, 2003; Baucheron *et al.*, 2004b; Chen *et al.*, 2007). Moreover, strains lacking components of the system showed increased susceptibility to various antimicrobial agents, with inactivation of *tolC* conferring hyper-susceptibility to a wider range of agents than inactivation of *acrB* (Sulavik *et al.*, 2001; Baucheron *et al.*, 2002; Baucheron *et al.*, 2004a; Eaves *et al.*, 2004b; Buckley *et al.*, 2006; Nishino *et al.*, 2006). However, when components of other *S. enterica* RND systems were inactivated (*acrD* and *acrF*) the effect on susceptibility was much less marked although this did result in over-expression of *acrB* (Eaves *et al.*, 2004b).

The AcrAB-TolC system of *Salmonella* is very similar to that in *E. coli* (94%, 97% and 94% similarity for *acrA*, *acrB* and *tolC*, respectively) but its structure and function have been more widely studied in *E. coli* (Table 1.2). Therefore, the following text focuses on work with *E. coli*. However, due to the significant DNA sequence, protein homology and similarities in effects upon antibiotic resistance in mutants it is reasonable to assume that the protein structure and pump mechanism is highly relevant to *Salmonella* (Piddock, 2006b).

1.8.1 TolC

TolC is a protein channel located in the outer membrane responsible for the expulsion of many compounds into the extracellular space. TolC, and members of the protein family to which it belongs, are ubiquitous throughout Gram-negative bacteria (Eswaran *et al.*, 2004; Koronakis *et al.*, 2004). Some of these outer membrane factors (OMFs), such as VceC of *Vibrio cholerae*, can only function with a specific PAP-RND pump (Federici *et al.*, 2005) while others, such as TolC, act as the outer membrane channel to multiple RND pumps, including AcrD (Hirakawa *et al.*, 2003; Nishino *et al.*, 2003), AcrEF (Nishino *et al.*, 2003), MdsAB (Nishino *et al.*, 2006) and MdtABC (Nagakubo *et al.*, 2002), as well as for the MFS systems EmrAB and EmrKY (Nishino and Yamaguchi, 2002) and also the ABC drug transporter MacAB (Kobayashi *et al.*, 2001b). Furthermore TolC can functionally replace VceC of *Vibrio cholerae* to function with the VceAB pump but VceC cannot functionally interact with AcrAB of *E. coli* (Vediyappan *et al.*, 2006).

Crystal structures have revealed that TolC is composed of three identical protomers each consisting of 428 residues (Figure 1.3 a). Each protomer has a 40Å long β -barrel domain anchored to the outer membrane and a 100Å long α -helical domain consisting of 12 coiled

Table 1.2 Percentage identity and percentage similarity to homologues of AcrAB-TolC

		% Identity	% Similarity
<i>S. Typhimurium</i>	<i>E. coli</i> K12		
AcrA	AcrA	91	94
AcrB	AcrB	94	97
TolC	TolC	89	94
<i>E. coli</i> K12	<i>P. aeruginosa</i>		
AcrA	MexA	56	71
AcrB	MexB	69	83
TolC	OprM	18	35
<i>E. coli</i> K12	<i>Campylobacter jejuni</i>		
AcrA	CmeA	31	51
AcrB	CmeB	41	63
TolC	CmeC	24	41

Data from Piddock, 2006a

coils which project into the periplasm. Together the monomers form an outer membrane pore and a periplasmic tunnel which projects into the periplasm and narrows to a closed end approximately 2 Å in diameter (Koronakis *et al.*, 2000; Koronakis *et al.*, 2004). The channel is permanently open on the extracellular surface but is restricted on the periplasmic side by di-sulphide bridges between cysteine residues on the coiled coils (Eswaran *et al.*, 2004). The channel is opened when the coiled coil helices move to the periphery of the channel by untwisting in an iris like movement (Koronakis *et al.*, 2000; Andersen *et al.*, 2002).

1.8.2 AcrB

AcrB is the RND efflux pump component of AcrAB-TolC and is responsible for the substrate specificity and energy transduction of the system (Elkins and Nikaido, 2002). The crystal structure of AcrB has been solved and shows that AcrB is a complex of three identical protomers forming an integral membrane protein complex within the cytoplasmic membrane that is “jellyfish shaped” (Murakami *et al.*, 2002) (Figure 1.3 c). Each protomer is composed of 1049 amino acids and has three domains: a transmembrane domain, a porter domain and a periplasmic TolC docking domain (Murakami *et al.*, 2006; Seeger *et al.*, 2006) (Figure 1.3 c).

The trans-membrane domain is formed by 12 membrane spanning α -helices. The fourth and tenth trans-membrane domains contain a triplet of charged amino acid residues, Asp 407, Asp 408 and Lys 940. These are vital for the utilisation of the proton motive force and are in a spatially separate location from the substrate binding pocket (Murakami *et al.*, 2006; Takatsuka and Nikaido, 2006; Seeger *et al.*, 2009). The trans-membrane domains of AcrB and MexB are particularly well conserved with almost all residues being identical, including the

three amino acids involved in this putative proton translocation site suggesting this may be a mechanism common to all RND systems (Sennhauser *et al.*, 2009).

There are two large periplasmic loops protruding from the transmembrane domain formed between the first and second, and seventh and eighth transmembrane domains each consisting of around 300 amino acids. The importance of the periplasmic loops was shown using chimeric studies in which, the loops from AcrB were replaced with the highly similar loops from AcrD in *E. coli*. The substrate specificity of the chimeric protein resembled that of AcrD, not AcrB, suggesting that the periplasmic loops are involved in substrate specificity (Elkins and Nikaido, 2002; Tikhonova *et al.*, 2002). Furthermore, work with another RND efflux pump of *E. coli*, YhiV, which has significant homology to AcrB, has shown that a mutation in the periplasmic loop region affected susceptibility to different antimicrobials in different ways. For example, a mutation which changed the valine residue at position 610 to a phenylalanine, increased resistance to linezolid and tetracycline but increased susceptibility to PA β N and azithromycin indicating further complexity in the substrate specificity imposed by the periplasmic loops (Bohnert *et al.*, 2007).

The porter domain of each protomer is made up of four subdomains, PN1, PN2, PC1 and PC2, arranged such that their β -sheets create the substrate binding pocket rich in aromatic residues, particularly phenylalanines, that interact with the structurally varied substrates (Murakami *et al.*, 2006; Seeger *et al.*, 2006). Crystallisation of AcrB in complex with bile acid showed that the binding site for this natural substrate is in a similar position to that for other published complexes including ethidium bromide, ciprofloxacin and PA β N (Yu *et al.*, 2005; Drew *et al.*, 2008). Site directed mutagenesis was used to show that mutation of different

phenylalanine residues within the binding pocket had variable effects on susceptibility to substrates and highlighted the F610 residue as having a particularly significant and broad impact on susceptibility (Bohnert *et al.*, 2008). Conversely, a substitution at the AcrB 616 residue was found to be particularly relevant for macrolide resistance (Wehmeier *et al.*, 2009) and a serine 715 substitution had a specific effect on bile resistance (Drew *et al.*, 2008). Redundancy of phenylalanines in the pocket may partially account for the varied consequences of substituting different residues. Some substrates are still accommodated in a mutated binding pocket while other larger substrates, such as the macrolides, are unable to adapt to changes caused by a single substitution (Bohnert *et al.*, 2008).

Loops which protrude from the periplasmic TolC docking domain into adjacent monomers are responsible for holding the three monomers together (Seeger *et al.*, 2008a; Sennhauser *et al.*, 2009).

Although the composition of each monomer is identical, three different teams have independently shown that the structure of AcrB is asymmetric. Each of the monomers are found in one of three different conformations and conformational cycling is responsible for the pumping mechanism of AcrB (Murakami *et al.*, 2006; Seeger *et al.*, 2006; Sennhauser *et al.*, 2007; Seeger *et al.*, 2008b; Takatsuka and Nikaido, 2009) (discussed further in section 1.10).

1.8.3 AcrA

AcrA is the periplasmic adaptor protein component of AcrAB-TolC and has been shown to physically interact with both AcrB and TolC (Zgurskaya and Nikaido, 2000; Augustus *et al.*,

2004; Husain *et al.*, 2004; Tikhonova and Zgurskaya, 2004; Touze *et al.*, 2004; Stenberg *et al.*, 2005). The cross-linking efficiency between AcrA and AcrB was not dependant on the presence of TolC (Zgurskaya and Nikaido, 2000; Tikhonova and Zgurskaya, 2004; Touze *et al.*, 2004). Similarly, the efficiency of cross linking of AcrA and TolC was not dependent on AcrB (Augustus *et al.*, 2004; Touze *et al.*, 2004). Furthermore, linking of AcrB and TolC could be detected in the absence of AcrA although the interaction was weak (Touze *et al.*, 2004; Tamura *et al.*, 2005).

Sequence analyses have shown that AcrA is a lipoprotein and hydropathy plots have suggested there are no transmembrane domains. It is bound to the inner membrane via an N-terminal lipid moiety (Ma *et al.*, 1993). A partial crystal structure of AcrA from *E. coli* was published by Mikolosko and colleagues in 2006. It was found to be 105 Å long and sickle shaped, in a similar manner to MexA, its homologue in *Pseudomonas aeruginosa* (Akama *et al.*, 2004; Higgins *et al.*, 2004; Mikolosko *et al.*, 2006). Despite sharing 62% sequence identity and 73% similarity, AcrA and MexA were unable to complement each other's function (Tikhonova *et al.*, 2002). Partial crystal structures of AcrA and MexA revealed three distinct domains: a β -barrel, a central lipoyl domain and an α -helical coiled coil hairpin (Ip *et al.*, 2003; Akama *et al.*, 2004; Higgins *et al.*, 2004; Mikolosko *et al.*, 2006) (Figure 1.3 b). The β -barrel domain has six anti-parallel β strands with a short α -helix (Zgurskaya and Nikaido, 2000; Mikolosko *et al.*, 2006). The lipoyl domain connects the α -helical hairpin and the β -barrel domain and is highly conserved among the family (Zgurskaya *et al.*, 2008). The α -helical hairpin domain is the most varied region within the PAP family with some of the Gram negative members lacking this domain completely (Bunikis *et al.*, 2008; Zgurskaya *et*

al., 2008). The AcrA hairpin domain is 58 Å long and has been shown to be of great functional significance as deletion of the 74 residues which form the hairpin resulted in a 100 fold decrease in the amount of AcrA protein and susceptibility to a range of antimicrobial substrates (Stegmeier *et al.*, 2006). It was suggested that removal of the hairpin domain destabilised the protein, preventing a functional efflux complex from forming. It is the C-terminal of the α -helical hairpin of AcrA which interacts with an intramolecular groove between the inner and outer periplasmic coiled coils of the outer membrane channel, TolC (Bokma *et al.*, 2006; Stegmeier *et al.*, 2006; Lobedanz *et al.*, 2007). The complete structure of MexA and AcrA, published in 2009, is congruent with the earlier versions although it also includes the contribution of N and the C termini to the structure which was previously omitted due to the apparent disordered nature of this domain (Symmons *et al.*, 2009). The N terminus (residues 13-27) and C terminus (residues 262-339) together form the membrane proximal (MP) domain which is a compact β roll linked to the β barrel domain by a β -ribbon (Symmons *et al.*, 2009) (Figure 1.3 b).

The packing state and stoichiometry of AcrA *in vivo* has, until very recently, been unclear (Zgurskaya *et al.*, 2008). *In vitro*, the soluble form of the protein appeared to be monomeric but subsequent cross-linking studies by the same authors appeared to show that only oligomers of three AcrA monomers could form complexes with each AcrB monomer (Zgurskaya and Nikaido, 1999a, 2000). X-ray structure analysis suggested that four AcrA molecules were arranged as a dimer of dimers (Mikolosko *et al.*, 2006) and another modelling approach suggested that a total of six AcrA molecules were present in the complex with two binding to each TolC monomer (Stegmeier *et al.*, 2006). However,

evidence has been accumulating that three AcrA molecules are present in each functional AcrAB-TolC complex. It was previously suggested that AcrA exists as a trimer as seen in AcrB and TolC (Stenberg *et al.*, 2005; Lobedanz *et al.*, 2007). Recently, following the completion of the AcrA crystal structure, more evidence from modelling with the complete structures of the three components and validated by extensive cross-linking has supported a stoichiometry of 3 AcrB: 3 AcrA: 3 TolC (Symmons *et al.*, 2009) (Figure 1.2). Interestingly, this is congruent with an earlier study which predicted that AcrA would be trimeric based upon known structures and predicting the most energetically favourable stoichiometric composition (Fernandez-Recio *et al.*, 2004). However, this is in contrast with recent data suggesting that the stoichiometry of the interaction between MexA and OprM varies from 2 to 6 MexA molecules per OprM trimer (Reffay *et al.*, 2009). Two further putative RND systems have been described which include two different PAPs. The Tri-OpmH efflux system of *P. aeruginosa* required both TriA and TriB for full efflux function (Mima *et al.*, 2007). Furthermore, the ZrpADBC of *Serratia sp.* was found to encode another efflux pump with two PAPs, ZrpA and ZrpD, however, only ZrpD was required for efflux function (Gristwood *et al.*, 2008).

Importantly, conformational flexibility has been revealed in the structure of both AcrA (Ip *et al.*, 2003; Mikolosko *et al.*, 2006) and MexA (Vaccaro *et al.*, 2006). Conformational changes in AcrA were detected *in vitro* in response to acidic pH which is physiologically relevant because drug efflux is linked to pH fluctuations in the periplasm which could trigger changes in AcrA structure (Ip *et al.*, 2003). Subsequently crystallisation revealed four different AcrA conformations (Mikolosko *et al.*, 2006). The flexibility is thought to come predominantly

from the hinge between the lipoyl domain and the α -helical hairpin and β -barrel domains and is reversible. Further flexibility was revealed when the crystal structure of AcrA was completed as the newly modelled MP domain was shown to rotate (Symmons *et al.*, 2009).

1.8.4 A fourth component: YajC

Crystal structures of endogenous AcrB complexed with a novel single transmembrane protein have recently been reported (Tornroth-Horsefield *et al.*, 2007). Endogenous AcrB was successfully crystallised and the structure solved by X-ray diffraction. This predicted that AcrB formed a complex with a previously undescribed protein termed YajC once the AcrAB-TolC efflux complex had assembled, inducing a functionally significant rotation of the porter domain of AcrB relative to the transmembrane domain. Deletion of YajC resulted in only a modest increase in susceptibility to ampicillin and nafcillin. Tornroth-Horsefield also suggested that the N-terminus of YajC could interact with AcrA and that AcrA could transmit the rotation in AcrB to TolC, facilitating the opening of the channel (Tornroth-Horsefield *et al.*, 2007).

1.9 Interactions between components of AcrAB-TolC

The interaction between AcrA and TolC is between the α -helical hairpin domain of AcrA and the intra-molecular groove between the inner and outer periplasmic coiled coils of the outer membrane channel, TolC, and the lipoyl and β -barrel domains of AcrA project away from the TolC complex (Bokma *et al.*, 2006; Stegmeier *et al.*, 2006; Lobedanz *et al.*, 2007). Importantly this model allows sufficient room for the movement of TolC during transition to its open state (Lobedanz *et al.*, 2007). Similar observations have also been made for the interactions

between the PAPs MexA or MexE with the outer membrane proteins OprN or OprM showing that this is representative of the RND family as a whole and not unique to AcrAB-TolC (Eda *et al.*, 2006; Stegmeier *et al.*, 2006).

The inner membrane domain of AcrB extends into the periplasm and interacts with the coiled coils of TolC. It was shown to interact with TolC by site-directed di-sulphide cross linking between cysteine residues on the tips of the hairpins of AcrB and the ends of the coiled coils of TolC (Tamura *et al.*, 2005) although there is some evidence that AcrA is required to stabilise this interaction *in vivo* (Touze *et al.*, 2004; Lobedanz *et al.*, 2007; Symmons *et al.*, 2009).

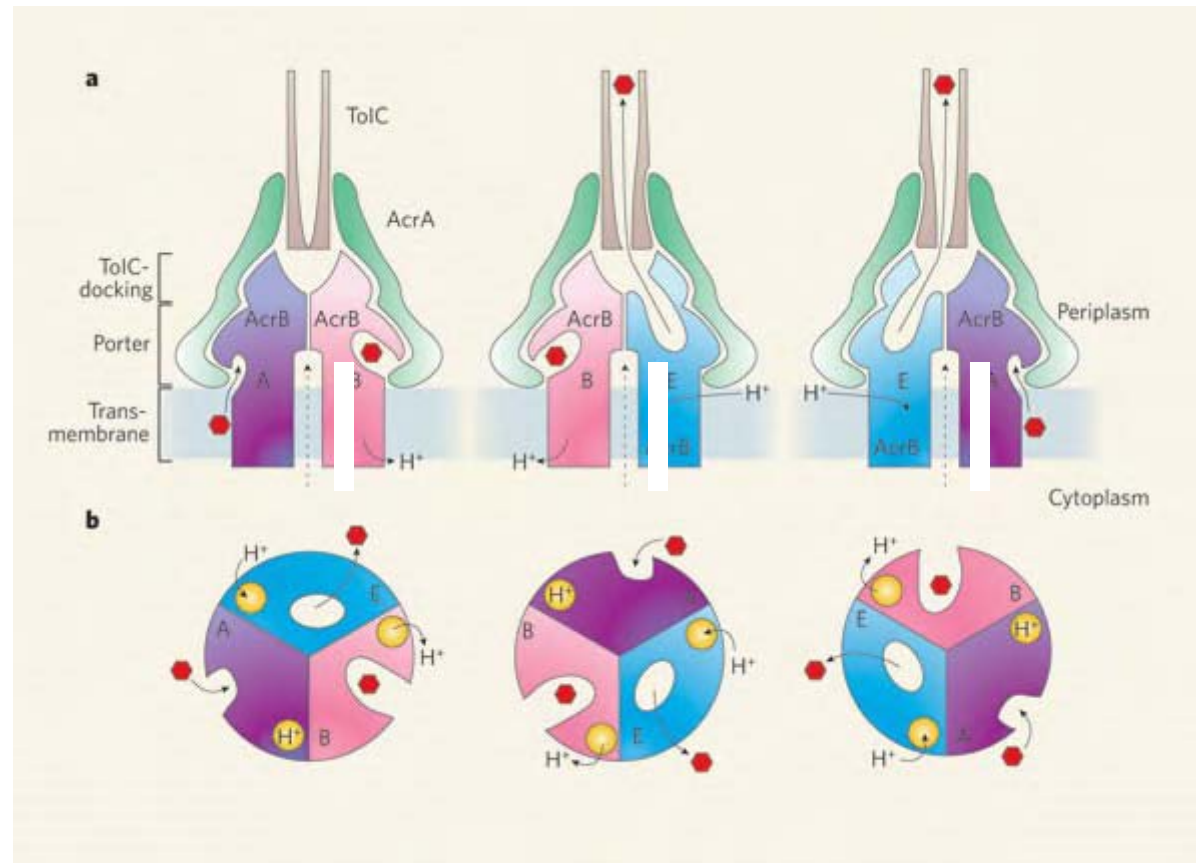
The interaction between AcrA and AcrB occurs independently of TolC or a substrate (Zgurskaya and Nikaido, 2000). It has been shown for both MexA and AcrA, that C-terminal is involved in interaction with the RND pump (Elkins and Nikaido, 2003; Nehme *et al.*, 2004; Nehme and Poole, 2007). The membrane proximal (MP) domain of AcrA interacts with the porter subdomains PN2 and PC1 while the β -barrel domain contacts the upper sections of these domains and also the docking domains DN and DC. The lipoyl domain of AcrA binds between the two halves of the TolC docking domain while the α -helical hairpin extends upwards to interact with TolC (Symmons *et al.*, 2009). The surface of the PN2, PC1, DN and DC subdomains of AcrB, which are closely associated with AcrA, are relatively stable and do not undergo large conformational changes associated with the functionally rotating mechanism which has been described for AcrB (Murakami *et al.*, 2006; Seeger *et al.*, 2008a). On the periplasmic surface the largest conformational change comes from the PC2 domain which is spatially separated from the AcrA attachment site. Symmons and colleagues (2009)

suggest this indicates that once docked, the conformational change required of the PAP would be limited although the flexibility is probably required for assembly of the pump (Symmons *et al.*, 2009).

1.10 Mechanism of transport by AcrAB-TolC

The understanding of the mechanism of action of the AcrAB-TolC efflux pump has advanced enormously in the last decade. In 2006, two teams showed that the three protomers of AcrB each exist in one of three different conformations at any one time and that this structure is central to its operation (Murakami *et al.*, 2006; Schuldiner, 2006; Seeger *et al.*, 2006). Murakami and colleagues referred to the three protomer conformations as access, binding and extrusion and suggest that AcrB functions in a three step binding change mechanism beginning with the substrate entering the protomer in the access state from the periplasm (Murakami *et al.*, 2006) (Figure 1.4). At this point it is thought that the substrate binds to the inside of the binding pocket which then expands in size allowing the substrate to move inside, the protomer is then in the binding conformation. At this stage the exit from the channel, into the TolC funnel, is blocked by an inclined helical domain. The protomer enters the extrusion state as the entry to the binding pocket is closed, the substrate moves through the channel binding to different positions in the pocket and the exit is opened as the helix moves away from it. The bound substrate is then finally pushed out of the binding pocket and into the funnel towards TolC (Murakami *et al.*, 2006). The binding pocket contracts as the subdomains PN1 and PC2 close (Sennhauser *et al.*, 2007). As previously mentioned the conformational changes are powered by the proton motive force across the membrane through the protonation and de-protonation of three charged residues, Aspartate-407,

Figure 1.4 The proposed functionally rotating mechanism of the AcrB efflux pump of *E. coli* (Adapted from Schuldiner, 2006)



- a. The side view of the AcrAB-TolC complex during drug transport. The substrate is depicted as a red hexagon.
- b. The proposed functionally rotating mechanism of AcrB. The drug enters the AcrB pump while it is in the 'access' conformation (purple and denoted by A) and binds to the complex in its 'binding' conformation (pink and denoted by B) and then exits the pump once it is in the extrusion conformation (blue and denoted by E) and the substrate is pushed towards the outer membrane channel, TolC. These conformational changes are powered by utilisation of the proton motive force.

Aspartate-408 and Lysine-940 in the transmembrane domain of AcrB (Murakami *et al.*, 2006; Seeger *et al.*, 2006; Takatsuka and Nikaido, 2006)t.

Biochemical evidence to support the functionally rotating mechanism has recently been published. Seeger and colleagues (Seeger *et al.*, 2008b) introduced constraining di-sulphide bonds between sub-domains of AcrB; these inhibited movement and pump function and increased susceptibility to toxic compounds. This effect was reversed after exposure to the reducing agent dithiothreitol, removing the constraints (Seeger *et al.*, 2008b). Takatsuka and Nikaido (Takatsuka and Nikaido, 2009) took the novel approach of creating a single “giant gene” containing three *acrB* sequences connected by short linker sequences enabling inactivation of a single protomer in the trimeric complex. Inactivation of one protomer abolished the activity of the whole complex confirming that the AcrB trimer is the functional unit and providing strong evidence for the functionally rotating hypothesis (Takatsuka and Nikaido, 2009).

From AcrB, the substrate must then progress through the outer membrane channel, TolC. The mechanism of opening of TolC was elucidated by removal of individual connections between α -helices of the same monomer and between different monomers which gradually increased conductance across the membrane representing slight relaxation of the tightly packed helices obstructing the entrance to the pore. Furthermore, elimination of multiple connections allowed the pore to open with the helices rearranging in an iris like movement (Andersen *et al.*, 2002). *In vivo* support for this hypothesis comes from an opposing experimental strategy of creating a TolC channel that is locked in the closed conformation by covalent bonds which constrain the periplasmic aperture (Eswaran *et al.*, 2003). This did not

prevent assembly of the Type I export machinery but abolished TolC mediated export demonstrating that untwisting of the α -helices is vital to TolC function (Eswaran *et al.*, 2003). It has been postulated that *in vivo* this opening mechanism is triggered by recruitment of TolC by the inner membrane transporter which provides energy for the process (Thanabalu *et al.*, 1998; Balakrishnan *et al.*, 2001) and the conformation is then stabilised by the adaptor protein (Andersen *et al.*, 2002). More recently, it has been suggested that the iris opening analogy may actually be too simplistic to describe the TolC opening and that an additional peristaltic motion in the periplasmic tunnel may aid movement of the substrate along the tunnel (Vaccaro *et al.*, 2008).

Another proposed mechanism is that, once AcrA is bound to AcrB, it then recruits TolC to the complex causing it to open and then the TolC channel remains in a constitutively open state, allowing the high-throughput transport displayed by this system (Symmons *et al.*, 2009). The “data driven modelling” approach employed suggests that the interaction between TolC and AcrA was most favourable when TolC was in its open state and that closure of TolC is not required because substrates can pass straight from AcrB to TolC without leakage into the periplasm as the connection between the two components is successfully stabilised by AcrA (Symmons *et al.*, 2009).

1.10.1 The role of AcrA in transport by AcrAB-TolC

Various hypotheses have been put forward for the precise role of AcrA and its PAP homologues in transport but no consensus has been reached. Firstly there is evidence that AcrA and MexA are involved in the assembly of the AcrAB-TolC pump complex (Mokhonov *et al.*, 2004; Tikhonova and Zgurskaya, 2004; Touze *et al.*, 2004; Ge *et al.*, 2009; Symmons *et al.*,

2009). Touze and colleagues (2004) have shown that the PAP is required for assembly of an active pump complex and other groups have suggested that AcrA is required to bring the outer and inner membrane components into close proximity (Tikhonova and Zgurskaya, 2004; Touze *et al.*, 2004; Zgurskaya *et al.*, 2008).

The periplasmic adaptor protein component could also be required to stabilise the pump complex (Touze *et al.*, 2004; Lobedanz *et al.*, 2007; Symmons *et al.*, 2009). Symmons and colleagues, for example, suggest that three single AcrA molecules each bind to a single AcrB-TolC unit, stabilising the complex. In this model the link between the α -helical hairpin domain of AcrA and the coiled coils of TolC was most compatible when TolC was in the open-state and the authors suggest that AcrA could stabilise the open conformation in the assembled tripartite pump (Symmons *et al.*, 2009).

Other studies suggest that AcrA is involved in conveying conformational changes in AcrB and mediating the opening of the outer membrane channel or actively transducing energy from AcrB to TolC (Ip *et al.*, 2003; Mikolosko *et al.*, 2006; Vaccaro *et al.*, 2006; Bavro *et al.*, 2008; Krishnamoorthy *et al.*, 2008). The proposed mechanism for this involves protein-protein interactions between the coiled coil hairpin of AcrA, which has been demonstrated to have considerable conformational flexibility, and the coiled coils of TolC. However, it is as yet unclear whether the observed conformational changes in AcrA play an active role in opening the outer membrane channel to allow efflux or whether it in fact it is passive (Mikolosko *et al.*, 2006). Interestingly, the extent of conformational change demonstrated in AcrA is of a similar magnitude to that seen during the opening of the TolC channel leading to speculation that AcrA could facilitate the opening of the channel (Koronakis *et al.*, 2000; Andersen *et al.*,

2002; Ip *et al.*, 2003; Mikolosko *et al.*, 2006; Bavro *et al.*, 2008). Bavro and colleagues suggest a model in which sequential interplay from all three components is required. They propose that conformational change in an activated AcrB causes partial opening of TolC which exposes an inter-protomer groove, allowing AcrA to properly bind which conveys the energy from AcrB, driving the complete opening of the TolC channel (Bavro *et al.*, 2008). Recent evidence following the publication of the complete AcrA structure suggests that this may be unlikely as only limited conformational change is actually required of AcrA in their fully assembled model. While the authors accept that transmission of conformational change by the PAP is not precluded, they suggest it is unlikely as the adaptor protein is bound to areas of the AcrB which do not undergo significant conformational changes and put forward the hypothesis that, once fully assembled, the TolC channel remains constitutively open (Symmons *et al.*, 2009).

Several other periplasmic adaptor proteins have been shown to have a role in substrate binding although this process is not well understood (Balakrishnan *et al.*, 2001; Borges-Walmsley *et al.*, 2003; Bagai *et al.*, 2007). For example, the PAP EmrA of *E. coli* has been shown to have a drug binding site and the authors speculated that it may have a role in transferring substrates to the outer membrane channel (Borges-Walmsley *et al.*, 2003). The recently published structure of the assembled tri-partite AcrAB-TolC efflux pump of *E. coli* showed that the AcrA protein is not in contact with the cleft between the PC1 and PC2 subdomains which is important for substrate access but the authors point out that a hydrophobic binding pocket between the PN2 and PC1 subdomains is very close to the site

of binding of the PAP membrane proximal domain and therefore a role for the PAP in substrate cannot be ruled out (Symmons *et al.*, 2009).

Finally, it has also been suggested that AcrA is able to modulate the activity level of the RND pump. The first evidence for this was presented by Zgurskaya and colleagues (1999) who demonstrated that transport and energy consumption by purified AcrB reconstituted into proteoliposomes was dramatically increased by addition of AcrA (Zgurskaya and Nikaido, 1999b). Furthermore, experiments with purified AcrD (a homologue of AcrB) reconstituted into proteoliposomes showed that it could transport aminoglycoside antibiotics, in a pH dependent fashion, but that activation by AcrA was required for this process (Aires and Nikaido, 2005). The PAP MtrC from *Neisseria Gonorrhoeae* has been shown to have a role in activating the RND pump, MtrD (Zhang *et al.*, 2009).

1.11 Genetic organisation of AcrAB-TolC

The *acrA* and *acrB* genes in *E. coli* and *S. Typhimurium* are found together in a single operon, separated by only 22 base pairs. The local repressor, *acrR*, is also co-located with *acrAB* but is divergently transcribed (Ma *et al.*, 1996). The *tolC* gene is encoded separately on the genome. The *acrAB* locus of *E. coli* was first mapped in 1965 by analysis of acriflavine sensitive *E. coli* mutants (Nakamura, 1965).

In *E. coli* there are two transcription start sites, one upstream of *acrA*, from which transcription of the *acrAB* transcript is initiated. More recently another transcription start site has been described 282 base pairs upstream of *acrB* (and within *acrA*) from which transcription of *acrB* alone begins although experimental evidence for this in the literature is

limited (Gama-Castro *et al.*, 2008) (Figure 1.5). However, this is congruent with earlier data from this laboratory where a northern blot using an *acrB* probe showed two distinct bands suggesting the presence of two *acrB* transcripts of differential size (Webber, 2002). Both of the transcriptional start sites described are conserved in *Salmonella* suggesting the mechanism is likely to be the same.

The ribosomal binding sites upstream of *acrA* and *acrB* have not been experimentally defined. The lack of a defined second ribosomal start site upstream of *acrB* means that translational coupling remains a possibility. Translational coupling is the sequential translation of the products of multiple genes, found in the same operon, without the need for the ribosome to disengage. Instead the ribosome terminates translation of one gene and then immediately initiates translation of the next. Translational coupling allows the ratio of two related gene products to be maintained and removes the requirement for a second ribosomal binding site (Inokuchi *et al.*, 2000; Saier, 2008).

1.12 Control of AcrAB-TolC expression

Expression of efflux pump genes is subject to multiple levels of regulation by a complex array of regulatory genes (Figure 1.6). The *acrAB* genes are regulated at a local level by the product of the *acrR* gene which represses transcription of *acrAB* (Ma *et al.*, 1996). AcrR is a member of the TetR family of transcriptional regulators which have a helix-turn-helix DNA binding motif at the N-terminal and a ligand binding domain at the C-terminal. The *acrR* gene is located 141 bp upstream of the *acrA* gene and is transcribed divergently (Figure 1.5 and 1.6). It was shown in *E. coli* that the AcrR protein prevents transcription of *acrAB* by binding to a 24 bp palindromic inverted repeat sequence between the *acrR* and

Figure 1.5 The genetic organisation of *acrAB* in *E. coli*

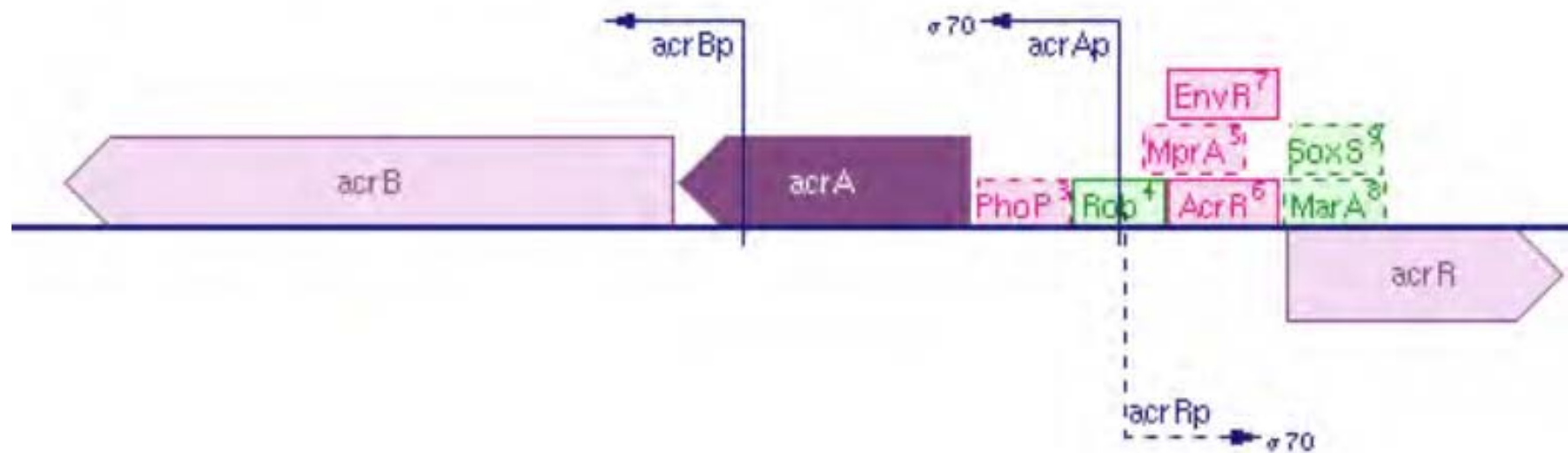


Diagram from www.ecocyc.org Green boxes represent transcriptional activators. Pink boxes represent transcriptional repressors.

Figure 1.6 The regulation of *acrAB-tolC* transcription

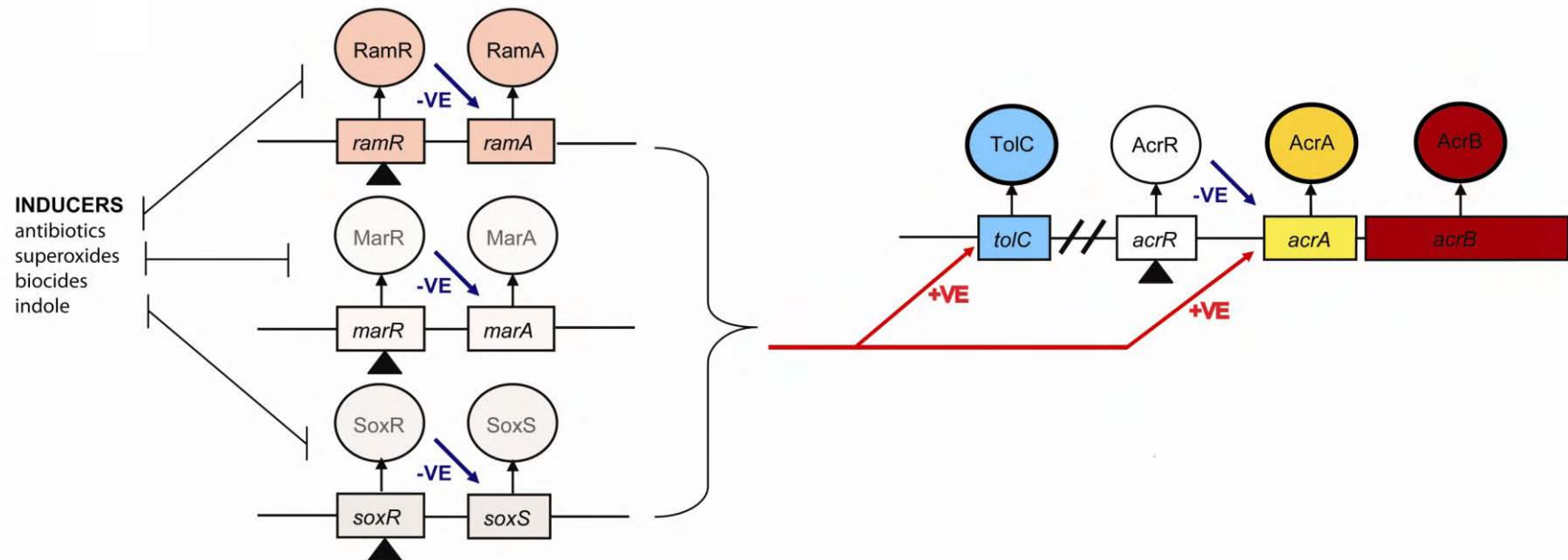


Figure courtesy of Dr Mark Webber, University of Birmingham

acrA genes which overlaps with the *acrAB* promoter sequence (Su *et al.*, 2007b). It is thought that transcription of *acrAB* is achieved when ligands such as ethidium bromide bind to the C-terminal of AcrR, causing conformational changes in the regulatory protein. This results in dissociation of AcrR from its target DNA sequence allowing transcription of the *acrAB* genes. AcrR and AcrB respond to the same ligands with very similar affinities so, at the local regulatory level, presence of a pump substrate, such as ethidium bromide, acts as a ligand to the local repressor protein, allowing transcription of *acrAB* and subsequent production of the efflux system which pumps out the toxic substance (Su *et al.*, 2007a; Su *et al.*, 2007b). In *Salmonella enterica* serovar Choleraesuis a mutation in the *acrR* gene, causing insertion of a stop codon, resulted in over-expression of *acrAB* and this contributed to ciprofloxacin resistance (Chiu *et al.*, 2005).

AcrAB is also regulated at the global level where the predominant regulators in *S. enterica* are the RamA, MarA, SoxS and Rob which are members of the XylS/AraC family of transcriptional regulators. RamA is a transcriptional activator of *acrAB* found in *S. enterica*, *Enterobacter cloacae* and *Klebsiella pneumoniae* but not in *E. coli* (van der Straaten *et al.*, 2004; Ruzin *et al.*, 2005; Keeney *et al.*, 2007). RamA is responsible for causing up-regulation of transcription of *acrAB-tolC* in response to environmental signals such as indole by binding to sites upstream of both *acrAB* and *tolC* (Nikaido *et al.*, 2008). When *ramA* was inactivated, expression of *acrB* was reduced but antibiotic susceptibility was unaffected (van der Straaten *et al.*, 2004; Bailey *et al.*, 2008). High level over-expression of *ramA* in *S. Typhimurium* led a 15 fold increase in expression of *acrB* and conferred resistance to nalidixic acid, chloramphenicol, tetracycline and triclosan (Bailey *et al.*, 2008). Over-expression of *ramA*

has also been detected in MDR laboratory mutants and clinical and veterinary isolates where it is linked with increased expression of AcrAB, AcrEF and MdtABC (Giraud *et al.*, 2000; Piddock *et al.*, 2000; Abouzeed *et al.*, 2008; Bailey *et al.*, 2008; Feuerriegel and Heisig, 2008; O'Regan *et al.*, 2009; Zheng *et al.*, 2009). For example Zheng and colleagues (2009) recently reported that a nine bp deletion in the *ramA* promoter led to constitutive expression of *ramA*, which was directly associated with over-expression of *acrAB* and *acrEF* leading to an MDR phenotype (Zheng *et al.*, 2009).

Expression of *ramA* is controlled by the transcriptional repressor RamR. Inactivation of *ramR* led to over-expression of *ramA*, which caused four-fold over-expression of the *acrAB* and subsequent MDR (Abouzeed *et al.*, 2008). In addition, fluoroquinolone and quinolone resistant isolates of *S. Typhimurium* were shown to have non-synonymous point mutations in *ramR* causing over-expression of AcrAB (Abouzeed *et al.*, 2008).

Unlike RamA, the transcriptional activators MarA, SoxS and Rob are found in both *Salmonella* and *E. coli* between which they share significant homology and function. SoxS is another member of the AraC/Xyls family of transcriptional regulators and is responsible for activation of at least a dozen genes involved in the response to oxidative stress including *acrB* (White *et al.*, 1997). SoxS is regulated by SoxR which is co-located with *soxS* on the genome. The *soxS* and *soxR* genes are separated by only 85 bp and are divergently transcribed (Amabile-Cuevas and Demple, 1991). The SoxR protein is activated by either oxidation (Nunoshiba *et al.*, 1992) or nitrosylation (Ding and Demple, 2000) causing it to induce expression of SoxS and subsequent expression of *acrAB*. In *E. coli* increased expression of *soxS* was shown to contribute to MDR in six post-therapy isolates (Webber and

Piddock, 2001). Furthermore, a similar effect was caused by a mutation in *soxR* causing constitutive expression of both *soxR* and consequently *soxS*, which contributed to the multiple antibiotic resistance phenotype of three clinical isolates (Koutsolioutsou *et al.*, 2005). Constitutive expression of *soxRS* can also contribute to MDR in *Salmonella*. A post therapy clinical isolate of *S. Typhimurium* had a point mutation in the *soxR* gene, causing constitutive expression and consequent quinolone resistance (Koutsolioutsou *et al.*, 2001).

The multiple antibiotic resistance or *mar* locus of *E. coli* and *Salmonella* consists of the genes *marA*, *marB*, *marC* and *marR*. MarA is a DNA binding transcriptional activator which controls the expression of over 60 genes in *E. coli*, including *acrAB* and *tolC*, by binding to a consensus sequence called the 'marbox' close to the promoter of genes in the regulon (Okusu *et al.*, 1996; Martin *et al.*, 1999; Barbosa and Levy, 2000). Inactivation of *marA* in antibiotic resistant strains of *E. coli* abolished the resistance phenotype (Cohen *et al.*, 1993). Furthermore, loss of function mutations in the local repressor, *marR*, led to over-expression of *marA* and to increased resistance to various antibiotics (Maneewannakul and Levy, 1996; Okusu *et al.*, 1996).

Expression of *E. coli marA* in *S. Typhimurium* conferred the multiple antibiotic resistance phenotype, showing the similarity of the Mar protein and regulon in the two species (Sulavik *et al.*, 1997). Elevated levels of MarA were detected along with increased expression of AcrB, AcrD and AcrF in MDR post therapy isolates of *Salmonella Typhimurium* and inactivation of *acrB* or *acrF* caused a compensatory upregulation of *marA* (Eaves *et al.*, 2004b)

Another member of the XylS/AraC family, Rob, is a constitutively expressed and involved in organic solvent tolerance. In *E. coli* over-expression of Rob, was reported to cause an

increase in antimicrobial resistance and up-regulation of *acrAB* by 1.9 fold (Linde *et al.*, 2004). Deletion *rob* increased susceptibility to organic solvents while over-expression led to resistance and moderate levels of drug resistance, due to slight over-expression of AcrAB-TolC (White *et al.*, 1997; Bennik *et al.*, 2000).

Recently another regulatory gene, *acrS*, which is located upstream of *acrEF*, has also been shown to have a regulatory effect on *acrAB* (Hirakawa *et al.*, 2008).

1.13 Promiscuity of AcrAB-TolC components

Although the components of the AcrAB-TolC pump function together as a tri-partite pump, this is not the only context in which the individual components can function. For example In *E. coli*, TolC has been shown to be required for the function of at least seven other efflux systems. These include the RND pumps AcrD (Hirakawa *et al.*, 2003; Nishino *et al.*, 2003), AcrEF (Nishino *et al.*, 2003), MdsAB (Nishino *et al.*, 2006) and MdtABC (Nagakubo *et al.*, 2002), as well the MFS systems EmrAB and EmrKY (Nishino and Yamaguchi, 2002) and the ABC drug transporter MacAB (Kobayashi *et al.*, 2001b). Over-expression of *acrD* in a strain lacking functional AcrB conferred a significant rise in β -lactam resistance but this resistance was not seen when *acrD* was over-expressed in a strain which also lacked *tolC* suggesting that TolC was required for the function of this pump (Nishino *et al.*, 2003).

AcrA has also been shown to associate with at least three other transporters. These include AcrD (Elkins and Nikaido, 2002, 2003), AcrF (Kobayashi *et al.*, 2001a; Elkins and Nikaido, 2003) and MdtF (Elkins and Nikaido, 2003). The ability of AcrD to efflux amphiphilic substrates, such as novobiocin and bile salts, was shown to be dependent on the presence of

AcrA. Furthermore, cross-linking studies followed by immunoblotting with anti-AcrB and anti-AcrA antibodies showed that AcrA can form stable complexes with both AcrB and AcrD (Elkins and Nikaido, 2002). Both the promiscuous interactions described have been confirmed by Hirakawa and colleagues (Hirakawa *et al.*, 2003) who showed that over-expression of AcrD in a $\Delta acrB$ background gave resistance to novobiocin but when AcrD was over-expressed in either a $\Delta acrAB$ or $\Delta tolC$ background this increase in resistance was not seen. This implicates both AcrA and TolC in interactions with AcrD (Hirakawa *et al.*, 2003).

Further insights into the functional associations of pump components have been gained from experiments in which components of AcrAB-TolC from *E. coli* and the homologous MexAB-OprM system from *P. aeruginosa* were combined to make chimeric pumps (Stegmeier *et al.*, 2006; Krishnamoorthy *et al.*, 2008). *E. coli* AcrAB will form a non-functional complex with the outer membrane channel of *P. aeruginosa*, OprM (Stegmeier *et al.*, 2006). Furthermore, a partly functional chimera consisting of AcrA with MexB and TolC can be formed, giving resistance to some, but not all, MexB substrates (Krishnamoorthy *et al.*, 2008). Mutations were identified which restored full functionality to the chimeric pump and as a result it was suggested that the reduced functionality was probably due to misalignment between the foreign components. This chimeric approach shows that the functional association of efflux pump components contains a degree of flexibility. However, this flexibility is not complete as neither MexAB-TolC, MexA-AcrB-TolC nor AcrA-MexB-OprM combinations were functional (Krishnamoorthy *et al.*, 2008). Furthermore, while AcrB can be forced to make promiscuous interactions with efflux pump components other than AcrA

and TolC, there is no evidence that it does so naturally. However, AcrB has recently been shown to have a role in a completely separate process, contact-dependant growth inhibition (CDI), possibly as a downstream target for the CDI signal, and this is independent of both AcrA and TolC (Aoki *et al.*, 2008).

1.14 The Physiological Role of AcrAB-TolC

In addition to an established role in innate antimicrobial resistance, MDR efflux pumps, including AcrAB-TolC, have been shown contribute to the basic biology of various pathogens (Piddock, 2006b). Work in this laboratory has contributed to this theory. Various efflux mutants (including *acrB::aph* and *tolC::aph*) were constructed and the effect of the mutations on the antibiotic susceptibility and virulence was investigated (Eaves *et al.*, 2004a; Buckley *et al.*, 2006). Tissue culture experiments showed that *acrB* mutants retained their ability to adhere to human embryonic intestine cells (INT-407) but invaded poorly (Buckley *et al.*, 2006). Conversely, in mouse macrophages (RAW 264.7) the mutants lost the ability to invade *in vitro*. Mutants lacking *tolC* adhered poorly to both INT-407 and RAW 264.7 cells lines *in vitro* and could not invade the mouse macrophages (Buckley *et al.*, 2006). Electron microscopy revealed that the *acrB* and *tolC* mutants induced fewer membrane ruffles on the surface of infected INT-407 cells. Furthermore, *in vivo* experiments in the avian host (in collaboration with Professor Martin Woodward at the Veterinary Laboratories Agency in Surrey) showed that colonisation and persistence was significantly reduced in mutants lacking either *acrB* or *tolC*. Complementation ablated the mutant phenotypes in both cases (Buckley *et al.*, 2006). This work showed that the AcrB and TolC proteins are needed for adhesion and invasion of *S. Typhimurium* *in vitro* and colonisation and persistence in the

chicken so therefore have an important role in virulence. This work fitted well with a growing body of evidence that RND systems are required for the virulence of several species as mutants lacking functional RND efflux systems or components thereof are attenuated (Stone and Miller, 1995; Lacroix *et al.*, 1996; Hirakata *et al.*, 2002; Jerse *et al.*, 2003; Lin *et al.*, 2003; Burse *et al.*, 2004; Chan and Chua, 2005; Buckley *et al.*, 2006; Nishino *et al.*, 2006; Piddock, 2006b; Bina *et al.*, 2008; Bunikis *et al.*, 2008). AcrB was shown to be required for the intestinal colonisation of mice and also for resistance to detergents including bile (Lacroix *et al.*, 1996). Nishino and colleagues reported that *S. Typhimurium* lacking the AcrAB-TolC or MacAB efflux systems had attenuated virulence in the mouse model after intragastric inoculation and that when nine other drug efflux genes were deleted simultaneously the resulting strain was rendered completely avirulent in this model (Nishino *et al.*, 2006). AcrAB-TolC of the plant pathogen *Erwinia amylovora* is needed for virulence (Burse *et al.*, 2004) while MtrCDE (homologous to AcrAB-TolC) was required for survival of *Neisseria gonorrhoeae* in the female mouse genital tract model (Jerse *et al.*, 2003) and the MexAB-OprM mutants of *P. aeruginosa* had a reduced ability to invade Madin-Darby Canine Kidney (MDCK) cells (Hirakata *et al.*, 2002). More recently, the same team has shown that addition of the efflux pump inhibitor PA β N significantly reduced the invasive ability of *P. aeruginosa* in a concentration dependant manner (Hirakata *et al.*, 2009). *C. jejuni* lacking the CmeABC efflux pump (homologous to AcrAB-TolC), which mediates resistance to bile in the intestinal tract, could not colonize chickens. Furthermore, oral administration of the efflux pump inhibitor PA β N to chickens 2-4 days post inoculation with *C. jejuni* significantly reduced colonisation suggesting that the reduction in virulence was due, at least in part, to a reduction in efflux (Lin *et al.*, 2003; Lin and Martinez, 2006). Collectively, these data provide

compelling evidence these efflux systems are vital for pathogenicity and virulence of a range of Gram-negative bacteria.

The contribution of these efflux systems to pathogenicity can be partially explained by the finding that many MDR efflux pumps, or certain components thereof, are also responsible for exporting host derived substrates such as bile salts, steroid hormones and fatty acids allowing the bacteria to survive within the hostile host environment (Ma *et al.*, 1995; Thanassi *et al.*, 1997; Elkins and Mullis, 2006; Drew *et al.*, 2008). Deletion of *acrAB* in *E. coli* led to increased susceptibility to bile salts and decanoate (a fatty acid) and the expression of these genes were elevated in the presence of decanoate (Ma *et al.*, 1995; Rosenberg *et al.*, 2003; Nikaido *et al.*, 2008). Furthermore, expression of *acrAB* in *Salmonella* Typhimurium and *acrD*, *acrE*, *emrK*, *mdtA*, and *mdtE* of *E. coli* are induced by the presence of indole or bile, both of which are present in the human intestine, allowing these toxic compounds to be exported permitting bacterial survival within the host (Hirakawa *et al.*, 2005; Nikaido *et al.*, 2008). The affinity of AcrAB from *E. coli* to a range of substrates was measured following *in vitro* reconstitution of AcrAB into proteoliposomes. The results showed that AcrAB had a far greater affinity for bile acids than for the antibiotics tested and the authors suggest this shows that AcrB is better adapted for the efflux of bile acids and that this is the function for which it evolved (Zgurskaya and Nikaido, 1999b; White *et al.*, 2005). Numerous genome wide mutagenesis studies of *S. Typhimurium* have been reported which aimed to identify genes involved in virulence in the mouse model. Interestingly, in three studies which all used the intraperitoneal method of infection, none identified the AcrAB-TolC system as being important for virulence (Lawley *et al.*, 2006; Chaudhuri *et al.*, 2009; Santiviago *et al.*, 2009).

This suggests that, while these proteins appear to be important for oral infection, where *Salmonella* would have to survive the hostile environment of the gut and resist the antimicrobial effects of bile, they are not needed when inoculation is directly into the peritoneal cavity.

Another class of host derived molecules which are substrates of RND efflux pumps are the antimicrobial peptides (AMPs) (Shafer *et al.*, 1998; Tzeng *et al.*, 2005; Eswarappa *et al.*, 2008). AMPs are produced by various host cells including the epithelia of the gastrointestinal tract and exert their antimicrobial effect by insertion into the negatively charged bacterial membrane, creating channels which cause osmotic damage. The human AMP LL-37 and the porcine protegrin-1 are substrates of the MtrCDE complex of *Neisseria gonorrhoeae* leading to the hypothesis that efflux may have evolved as a mechanism to circumvent the innate antimicrobial defences of the host (Warner *et al.*, 2008). Furthermore, mutation of *mtrR*, which represses *mtrCDE* transcription, led not only to increased resistance to antimicrobials including host derived peptides, but also to an increase in fitness in the *in vivo* mouse model of infection suggesting that resistance to AMPs could contribute to virulence (Warner *et al.*, 2007; Warner *et al.*, 2008; Folster *et al.*, 2009). AMPs may not be substrates of all RND pumps or may have a species specific role as resistance to several AMPs was not affected by mutation of *acrAB* in *E. coli* or *mexAB* of *P. aeruginosa* (Rieg *et al.*, 2009). However, in contrast, another team have suggested that AcrAB-TolC along with regulators thereof are in fact involved in resistance to human AMPs including LL-37, Polymyxin B and β -defensins as deletion of *acrB* or *tolC* increased susceptibility to these AMPs (Warner and Levy, 2009).

Furthermore, over-expression of *marA* in the presence of AcrAB decreased susceptibility to these AMPs (Warner and Levy, 2009).

However, the transport of host derived molecules does not explain the attenuation seen *in vitro* as these host factors are largely absent. Hirakata and colleagues showed that the reduction in invasiveness seen in *P. aeruginosa* mutants lacking MexAB-OprM was restored not only by complementation of the disrupted genes but also by addition of culture supernatant from MDCK cells infected with wild-type *P. aeruginosa* (Hirakata *et al.*, 2002). This suggests that an exported factor, present in the supernatant, is able to ameliorate the adhesion and invasion defects in the mutant.

Alternatively, it has been proposed that the impaired ability of efflux mutants to cause infection could be due to altered expression of other genes or groups of genes that are linked to the ability to cause infection. For *Salmonellae* the attenuation due to lack of TolC has been associated with reduced expression of SPI-1 genes and subsequently reduced secretion of T3SS-1 effectors (Virlogeux-Payant *et al.*, 2008; Webber *et al.*, 2009). Likewise, inactivation of *acrB* led to reduced expression of SPI-1 genes and its effectors and other pathogenicity related genes (Webber *et al.*, 2009). Webber *et al.*, (2009) also showed that lack of functional AcrA was associated with decreased expression of genes found in SPI-2. Inactivation of *acrA*, *acrB* or *tolC* had different effects on the transcriptome. For example, expression of genes involved in anaerobic metabolism were increased in the absence of AcrA but decreased in the absence of AcrB although both strains had an impaired ability to grow in anaerobic conditions. Also, changes in motility gene expression was seen following inactivation of *acrB* or *tolC*, but not *acrA* though only the *acrB* mutant had reduced motility

compared to wild-type (Webber *et al.*, 2009). This suggests that the attenuation of virulence in strains lacking AcrA, AcrB or TolC could be mediated by different mechanisms.

Another suggestion is that AcrAB-TolC and its homologues are also responsible for the export of metabolic products (Helling *et al.*, 2002; Poole, 2005; Huang *et al.*, 2008; Rosner and Martin, 2009). Rosner and Martin recently hypothesised that in *E. coli* intracellular accumulation of toxic bacterial metabolites during growth led to up-regulation of MarA, SoxS and Rob, which stimulates production of the TolC protein to remove the metabolites (Rosner and Martin, 2009). This is in agreement with an earlier study by Helling and colleagues suggesting that AcrAB-TolC is required to export products of normal metabolism to prevent them building up to toxic levels (Helling *et al.*, 2002).

A further novel physiological role for AcrB in *E. coli* in contact-dependent growth inhibition (CDI) has been proposed that is independent of both AcrA and TolC (Aoki *et al.*, 2008). CDI is the regulation of growth via cell to cell contact and significant numbers of mutants with resistance to CDI showed mutations in *acrB* or *bamA*, the latter of which is a member of the Omp85 family of essential outer membrane proteins. The specific role of AcrB in this process has not been elucidated but it may be a downstream target for the CDI signal (Aoki *et al.*, 2008).

Other possible explanations for the involvement of efflux pumps in pathogenicity will be investigated during this project.

Hypotheses

The hypotheses to be explored in this project are:

- Inactivation of *acrA* will confer hypersusceptibility to a range of antimicrobial compounds.
- Inactivation of *acrA* will attenuate the ability of *S. Typhimurium* to infect INT-407 cells and RAW 263.7 cells *in vitro*.
- A virulence related factor is exported by AcrAB-TolC but is not exported, or not exported efficiently in the mutants lacking components of the system. Exogenous addition of this factor may be able to restore the virulence defect of the efflux mutants.

Aims and objectives

- To inactivate *acrA* in *Salmonella* Typhimurium and ensure that *acrB*/AcrB is still expressed.
- To characterise the effect of lack of AcrA on the growth, innate antimicrobial resistance profile, bacterial morphology and level of Hoechst H33342 accumulation (efflux activity).
- To determine the effect of lack of AcrA on the ability of SL1344 to infect INT-407 cells and RAW 264.7 *in vitro*.
- To determine whether a virulence related factor, exported by AcrAB-TolC in SL1344, is able to restore the invasiveness of the efflux mutants.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Bacterial strains, growth, storage and identification

Salmonella enterica serovar Typhimurium SL1344 (laboratory code, L354; Table 2.1) was used as the wild-type strain (Wray and Sojka, 1978). This strain was chosen because it is a pathogenic strain isolated from an experimentally infected calf. This means that aspects of pathogenesis and virulence can be studied using this strain unlike another common type strain, LT2, which is avirulent due to a mutation in the *rpos* gene (Wilmes-Riesenberg *et al.*, 1997). This strain has also been used for previous work on *Salmonella* in this and multiple other laboratories.

The *acrA* gene of SL1344 was inactivated by insertion of the *aph* gene, conferring kanamycin resistance, to give an *acrA* mutant with the laboratory code L823 (*acrA::aph*). The disrupted *acrA* region was transduced into SL1344 giving L884 (section 2.3), the *aph* gene was removed from L823 and L884 giving L976 and L978 (Section 2.4) and *acrA* was cloned into pWKS30 and transformed into L354, L823 and L884 giving L980, L975 and L979, respectively (Section 2.5) (Table 2.1). The phenotype of the *acrA* mutants was compared to other mutants previously constructed in this laboratory: L109 (*tolC::aph*; transductant of original mutant L108) and L110 (*acrB::aph*; transductant of original mutant L643) (Eaves *et al.*, 2004; Buckley *et al.*, 2006).

All strains were routinely grown on Luria-Bertani (LB) agar (Sigma-Aldrich Ltd., UK), supplemented with appropriate antibiotics where necessary, and incubated overnight at 37°C (Table 2.2). Overnight cultures were grown in LB broth (Sigma-Aldrich Ltd., UK), supplemented with appropriate antibiotics where necessary, and incubated overnight at 37°C with aeration. Where stated, minimal media (Teknova) was also used. Minimal media was prepared by adding 100ml of 10 x MOPs buffer (Teknova, M2101) to 10ml 20% Glucose (Teknova, G0520), 10ml 0.132M Potassium phosphate (Teknova, M2102). L- Histidine was also added to a final concentration of 45 mM because SL1344 is a

Table 2.1 Strains used during this study

Birmingham Code	Genotype/plasmid	Resistance Phenotype	Reference
L354	<i>S. enterica</i> serovar Typhimurium SL1344		Wray and Sojka, 1978
I633	<i>E. coli</i> K12 with pKD4	Amp ^R , Kan ^R	Datsenko and Wanner, 2000
L642	SL1344 with pKD46	Amp ^R	Datsenko and Wanner, 2000
L108	SL1344 <i>tolC::aph</i>	Kan ^R	Buckley <i>et al.</i> , 2006
L109	SL1344 <i>tolC::aph</i> Transduced from L108 to SL1344 using P22 phage	Kan ^R	Buckley <i>et al.</i> , 2006
L742	$\Delta tolC$ L108 treated with pCP20	Kan ^S	Eaves, unpublished data
L643	SL1344 <i>acrB::aph</i>	Kan ^R	Eaves <i>et al.</i> , 2004 Buckley <i>et al.</i> , 2006
L110	SL1344 <i>acrB::aph</i> . Transduced from L643 to SL1344 using P22 phage	Kan ^R	Eaves <i>et al.</i> , 2004 Buckley <i>et al.</i> , 2006
L644	$\Delta acrB$ L643 treated with pCP20	Kan ^S	Buckley <i>et al.</i> , 2006
L823	SL1344 <i>acrA::aph</i>	Kan ^R	This study
L884	SL1344 <i>acrA::aph</i> Transduced from L823 to SL1344 using P22 phage	Kan ^R	This study
L976	$\Delta acrA$ L884 treated with pCP20.	Kan ^S	This study
I906	<i>E. coli</i> K12 with pWKS30 vector	Amp ^R	Wang and Kushner, 1991
L917	SL1344 with pWKS30 vector	Amp ^R	This study

L918	L823 with pWKS30 vector	Amp ^R , Kan ^R .	This study
L996	L884 with pWKS30 vector	Amp ^R , Kan ^R	This study
L750	E. coli DH5α with pWKS30 <i>acrA</i>	Amp ^R	This study
L980	SL1344 with pWKS30 <i>acrA</i>	Amp ^R	This study
L975	L823 with pWKS30 <i>acrA</i>	Amp ^R , Kan ^R .	This study
L979	L884 with pWKS30 <i>acrA</i>	Amp ^R , Kan ^R	This study
L1019	SL1344 with pUA66pacpP	Kan ^R	This study
L1020	L644 (Δ <i>acrB</i>) with pUA66pacpP	Kan ^R	This study
L1021	L742 (Δ <i>tolC</i>) with pUA66pacpP	Kan ^R	This study
L1022	L976 (Δ <i>acrA</i>) with pUA66pacpP	Kan ^R	This study
kan, kanamycin; amp, ampicillin			

Table 2.2 Antibiotics, dyes and detergents used in this study

Agent	Solvent	Supplier
Ampicillin	Sodium Bicarbonate and Water	Fluka Biochemika, Sigma-Aldrich, UK
Kanamycin	Water	Fluka Biochemika, Sigma-Aldrich, UK
Ethidium Bromide	Water	Sigma-Aldrich, UK
Fusidic Acid	Water	Sigma-Aldrich, UK
Acriflavine	Water	Sigma-Aldrich, UK
Bile	Water	Fluka Biochemika, Sigma-Aldrich, UK
SDS (Lauryl Sulphate)	Water	Sigma-Aldrich, UK
Triclosan	70% Methanol	Sigma-Aldrich, UK
Nalidixic acid	Sodium Hydroxide and Water	Sigma-Aldrich, UK
Ciprofloxacin	Acetic Acid and Water	Sigma-Aldrich, UK
Chloramphenicol	70% Methanol	Sigma-Aldrich, UK
Tetracycline	Water	Sigma-Aldrich, UK
Gentamicin	-	Sigma-Aldrich, UK

histidine auxotroph. This mixture was made up to 1 L with H₂O and filter sterilised. Protect™ beads (Technical Service Consultants Ltd, UK) were used for long term storage. *Salmonella* strains were stored at -20°C and *E. coli* strains were stored at -80°C.

For identification purposes all bacteria were grown on *Salmonella*-selective Xylose Lysine Deoxycholate (XLD) agar (Sigma-Aldrich Ltd., UK) and MacConkey agar (Sigma-Aldrich Ltd., UK). The appearance of colonies with black centres on XLD suggested that the bacteria present were *Salmonella* while on MacConkey agar white/colourless colonies were indicative of *Salmonella*. The bacteria were all Gram stained and subsequently observed microscopically to ensure they were rod shaped Gram negative bacteria. Analytical profile index (API) testing, (Biomérieux, France) was used according to the manufacturer's instructions to check bacterial identity. Serotyping was performed at the Veterinary Laboratories Agency, Surrey.

2.2 Disruption of *acrA* in *S. Typhimurium*

The *acrA* gene was inactivated using the protocol described by Datsenko and Wanner (2000). This method causes disruption of chromosomal genes by homologous recombination with PCR products containing the *aph* gene whose product provides kanamycin resistance. This relies on the plasmid encoded λ red recombination system, which is based on the recombination systems found naturally in bacteriophages. In the first instance the *aph* gene was amplified from the pKD4 plasmid using primers that also have 40 bp of homology to the gene of interest. The second stage involves transforming the recipient strain, containing the pKD46 plasmid, with the PCR construct. Homologous recombination follows, mediated by the λ red recombinase system, to leave a mutant with the chromosomal gene of interest disrupted by the kanamycin resistance gene, *aph*. Mutants are then selected by growing on LB agar supplemented with 25µg/ml kanamycin (Datsenko and Wanner, 2000).

2.2.1 Isolation of the pKD4 plasmid

The pKD4 plasmid (Table 2.3) was isolated from *E. coli* strain I633. The strain was grown overnight at 30°C in LB broth with 50 µg/ml of ampicillin. The QIAprep® Miniprep Kit (QIAGEN, 27104) was used to harvest the plasmid, following the low copy number protocol to ensure maximum yield.

Agarose gels were prepared by adding 1 g of electrophoresis grade agarose (Invitrogen Ltd, UK, 15510-027) to 100 ml of 1% Tris-Boric acid-EDTA (TBE). The agarose was dissolved by heating in a 650 W microwave for approximately 90 seconds. The solution was allowed to cool before ethidium bromide was added to a final concentration of 0.1 µg/ml. The molten agarose was poured into a gel tray and allowed to set before being immersed in 1% TBE in the gel tank. Five micro litres of each sample was then loaded onto the gel along with a DNA sizing ladder of suitable size range (Bioline). Electrophoresis was used to separate the samples at 100 volts for one hour.

The quantity of plasmid collected was determined using the 'Gene genius' image analyser (Syngene, UK) and the Gene Tools software package (Syngene). The stock of isolated plasmid was stored at -20°C.

2.2.2 Generation of the PCR product to inactivate *acrA*

Primers 568 and 569 (Table 2.4) were designed using the PRIMER v.2.00 software (Scientific and Educational Software, UK). All primers were produced by Invitrogen (Invitrogen Ltd., UK) and are listed in Table 2.4. On arrival the primers were re-hydrated with UltraPure water (Invitrogen, UK) to a concentration of 100 µM and from this 25 µM working stock solutions of each primer were made. All primers were stored at -20°C.

Each primer had 40bp of homology to the target gene, *acrA*, 20bp of homology to the *aph* gene coding for kanamycin resistance which is found on the PKD4 plasmid and also a 3' GC clamp. The

Table 2.3 Plasmids used in this study

Plasmid	Description	Antibiotic Determinants	Resistance	Reference
pKD4	Used as PCR template to create construct for insertion into the gene of interest	Amp ^R . Kan ^R .		Datsenko & Wanner, 2000
pKD46	Encodes the λ red recombination system to aid homologous recombination.	Amp ^R .		Datsenko & Wanner, 2000
pCP20	Temperature sensitive plasmid which encodes the FLP gene whose product removes FLT-flanked antibiotic resistance determinants.	Amp ^R .		Datsenko & Wanner, 2000
pWKS30	Low copy number vector used for complementation	Amp ^R .		Wang & Kushner, 1991
pUA66pacpP	Plasmid which encodes <i>gfp</i>	Kan ^R		Neil Burton, personal communication

kan, kanamycin; amp, ampicillin

primers were designed to insert the *aph* gene between base pairs 297 and 1326 of the *acrA* gene causing juxtaposition of the 99th and 442nd codons. As the *acrB* gene is directly downstream of *acrA*, it was possible that removal of this section could cause polar effects which interfered with *acrB* transcription and subsequent translation to AcrB. Section 2.6.1 describes the method used for Reverse Transcriptase (RT) PCR to ensure *acrB* transcription was not affected while Section 2.6.2 describes the Western Blotting method used to check that the AcrB protein was translated at similar levels to wild-type.

PCR reactions were set up using 45 µl of 1.1 x ReddyMix™ PCR master mix (AB-0575-LD, ABgene, UK). In each reaction volume this master mix contains 0.625 units of *Taq* DNA polymerase, 75mM Tris-HCl, 20mM (NH₄)₂SO₄, 1.5mM MgCl₂, 0.01% Tween-20 and 0.2mM each of dATP, dCTP, dGTP and dTTP. 1.5 µl of a 25 µM stock of each of the inactivation primers and 2 µl of isolated pKD4 plasmid, as the template DNA, were added to the master mix. The initial denaturation step was carried out at 95°C for five minutes. This was followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds and elongation at 72°C for 2 minutes. This was followed by a final elongation stage at 72°C for ten minutes (Table 5, PCR 1).

The PCR product was purified using the QIAGEN® PCR Purification Kit (QIAGEN, 28106). The product was eluted in 30 µl of ultrapure water (Invitrogen) to ensure maximum DNA yield and minimum salt concentration to increase the chances of successful electroporation. The size of the purified PCR product was analysed by running the sample on a 1% agarose gel with Hyperladder 1 (Bioline, BIO-33025). This was electrophoresed at 100 volts for one hour and viewed on the 'gene genius' image

Table 2.4 Primers used in this study

Primer Number	Description	Sequence
568	<i>acrA</i> inactivation Forward	AGC GCT AAC AGG ATG TGA CGA CAA ACA GGA CCA GCA AGG C GT GTA GGC TGG AGC TGC TTC*
569	<i>acrA</i> inactivation Reverse	CTG TAC TTT AAC CTG TGC GCC AGG ACG TAC TTT TTG CAG CGG GAA TTA GCC ATG GTC CAT*
570	<i>acrA</i> Upstream Check	ACA TCC AGG ATG TGT TGT CG
571	<i>acrA</i> Downstream Check	CAA TCG TCG GAT ATT GCG CT
566	<i>acrA</i> Internal Check Forward	GCA GTA CAT CAG TAA GCA GG
567	<i>acrA</i> Internal Check Reverse	CCT TGC GTT ACG GAT GAC TT
324	16s Internal Forward	CCT CAG CAC ATT GAC GTT AC
325	16s Internal Reverse	TTC CTC CAG ATC TCT ACG CA

334	<i>acrB</i> Internal Check Forward	CGT GTT ATG ACG GAA GAA GG
335	<i>acrB</i> Internal Check Reverse	GCC ATA CCG ACG ACG ATA AT
237	Kt - Internal <i>aph</i> Reverse	CGG CCA CAG TCG ATG AAT CC
239	K2 – Internal <i>aph</i> Forward	CGG TGC CCT GAA TGA ACT GC
648	<i>acrA</i> complementation Forward	TCA AGC TTA CAT CCA GGA TGT GTT GTC G
649	<i>acrA</i> complementation Reverse	TCG GAT CCA TCG TCG GAT ATT GCG CTA C

* Highlighted areas correspond to the 20bp of homology to the kanamycin resistance cassette

Table 2.5 Parameters and primers used for each PCR reaction.

							PCR parameters (x 30 cycles)				
PCR Number	Primer 1			Primer 2			Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
1	568			569			95°C (5 min)	95°C (30 sec)	52°C (30 sec)	72°C (2 min)	72°C (10 min)
	<i>acrA</i> Forward	inactivation	<i>acrA</i> inactivation Reverse								
2	570			571			95°C (5 min)	95°C (30 sec)	52°C (30 sec)	72°C (2 min)	72°C (10 min)
	<i>acrA</i> Upstream check		<i>acrA</i> Downstream check								
3	566			567			95°C (5 min)	95°C (30 sec)	51°C (30 sec)	72°C (2 min)	72°C (10 min)
	<i>acrA</i> Internal Forward	Check	<i>acrA</i> Internal Reverse	Check							
4	334			335			95°C (5 min)	95°C (30 sec)	51°C (30 sec)	72°C (2 min)	72°C (10 min)
	<i>acrB</i> Internal Forward	Check	<i>acrB</i> Internal Reverse	Check							
5	324			325			95°C (5 min)	95°C (30 sec)	51°C (30 sec)	72°C (2 min)	72°C (10 min)
	16s Internal Forward		16s Internal Reverse								

6	570 <i>acrA</i> Upstream check	237 Internal <i>aph</i> Reverse	95°C (5 min)	95°C (30 sec)	52°C (30 sec)	72°C (2 min)	72°C (10 min)
7	239 Internal <i>aph</i> Forward	571 <i>acrA</i> Downstream check	95°C (5 min)	95°C (30 sec)	52°C (30 sec)	72°C (2 min)	72°C (10 min)
8	239 Internal <i>aph</i> Forward	237 Internal <i>aph</i> Reverse	95°C (5 min)	95°C (30 sec)	51°C (30 sec)	72°C (2 min)	72°C (10 min)
9	648 <i>acrA</i> Complementation Forward	649 <i>acrA</i> Complementation Reverse	95°C (5 min)	95°C (30 sec)	53°C (30 sec)	72°C (2 min)	72°C (10 min)

Primer information can be found in Table 2.4

analyser. The quantity of the PCR construct was determined using the Gene Tools software package (Syngene).

2.2.3 Transformation of the recipient Salmonella strain, L642, with the knockout PCR construct

A single colony of L642, the salmonella strain containing the λ red recombinase system on the pKD46 helper plasmid, was inoculated into LB broth, supplemented with 50 $\mu\text{g/ml}$ ampicillin and 10 mM arabinose, and grown overnight at 30°C with aeration (shaking at 150 rpm). The arabinose is required to induce the λ red recombination system on the pKD46 helper plasmid. 50 ml of pre-warmed SOB media containing 50 $\mu\text{g/ml}$ ampicillin and 10 mM arabinose was inoculated with 1 ml of the overnight culture and incubated at 30°C with aeration for approximately four hours until an OD_{600} of 0.6 was reached (mid logarithmic phase). The cells were harvested from this culture by centrifugation at 2250 x g for 10 minutes at 4°C in a Mistral 3000i. The cells were re-suspended in 50 ml of ice cold 10% glycerol to wash. This washing process was repeated a further three times. After the final centrifugation the pellet of competent L642 cells was re-suspended in 250 μl of ice-cold glycerol and left on ice.

Before electroporation a range of quantities (typically 50 ng, 100 ng and 150 ng) of the *aph* containing PCR construct were added to approximately 45 μl of competent cells to a total volume of 50 μl and put into a chilled 0.2 cm electroporation cuvette. The samples were electroporated at 2.5 kV (25 μF , 200 Ω). A negative control was also included which contained 50 μl of competent cells and no PCR product. After electroporation the cells were recovered in 1 ml of pre-warmed SOB media supplemented with 10 mM arabinose. This was incubated at 37°C with aeration (shaking at 150 rpm) for two hours at 37°C. Half of the recovery mixture was transferred in 200 μl quantities onto selective LB agar plates containing 25 $\mu\text{g/ml}$ kanamycin and incubated overnight at 37°C. The remaining cells were left to recover at room temperature overnight and then transferred in 200 μl quantities onto selective LB agar plates containing 25 $\mu\text{g/ml}$ kanamycin. This allows for selection of

transformants and for loss of the pKD46 helper plasmid as the plates do not contain ampicillin. The agar plates were incubated at 37°C for two days and examined for candidate recombinants.

2.2.4 Verification of gene disruption in candidate colonies by PCR

All colonies which appeared on the selective media after two days were sub-cultured onto separate LB agar plates containing 25 µg/ml kanamycin and incubated overnight at 37°C. Whole cell lysates were prepared by putting a loop full of bacterial culture from the agar plate into 100 µl of sterile water and heating to 99°C for 10 minutes.

Primers 570 and 571 were designed to allow the insertion of the *aph* gene to be checked by PCR (Table 2.4). The check primers had 20 bp of homology to regions upstream and downstream of the target insertion site for the *aph* gene. Four PCR reactions were performed from each candidate using combinations of the upstream and downstream check primers and primers internal to the *aph* gene (Table 2.4) as an initial screen for successful gene inactivation. The PCR reaction contained 45 µl of 1.1 x ReddyMix™ PCR master mix (AB-0575-LD, ABgene, UK), 1.5 µl of a 25 µM working stock of each primer and 2 µl of the whole cell lysate as the template. Wild-type genomic DNA was used as a control. The initial denaturation step of the PCR was five minutes at 95°C. For PCR reactions 2, 6 and 7 (Table 2.5) 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds and elongation at 72°C for 2 minutes were used. For PCR reaction 8 (Table 2.5) this was followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds and elongation at 72°C for 30 seconds. This was followed in both cases by a final elongation stage at 72°C for ten minutes. The sizes of the PCR amplicons were analysed by running the samples on a 1 % agarose gel along with Hyperladder 1 (Biolone, UK, BIO-33025). This was electrophoresed at 100 volts for one hour and viewed on the 'gene genius' image analyser. The size of amplicons produced from the candidates was compared to the amplicons size produced from SL1344 DNA and the predicted amplicons size. Amplicon sizes were predicted by accessing the wild-type sequence (from

<http://xbase.bham.ac.uk>) and calculating the size of the PCR fragment which would be produced before and after insertion of the *aph* gene.

2.2.5 Sequencing of candidate *acrA* mutants

Once transformation had been confirmed by the PCR reactions, the DNA upstream and downstream of the inactivated gene was sequenced to guarantee that the gene disruption had occurred as predicted. PCR reactions 6 and 7 (Table 2.5) were used to amplify the region to be sequenced in each candidate. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, 28106) and were eluted in 30 µl of water. The PCR product was separated by electrophoresis at 100 V for approximately one hour on a 1 % agarose gel electrophoresis. The amount of DNA was quantified using the 'Gene Tools' software (Syngene) using HyperLadder I (Bioline) as a reference for quantity. The sequencing reaction was performed by the Functional genomics and Proteomics unit at the University of Birmingham using the BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems Ltd., U.K) following the protocol described by ExGen, Functional Genomics and Proteomics Unit (School of Biosciences, University of Birmingham). The sequence data was aligned and compared with SL1344 and the expected sequences using the GeneDoc software package.

2.2.6 Verification that transformants were *S. enterica*

To confirm bacterial identity, each of the candidate transformants were sub-cultured onto Salmonella-selective XLD agar (Sigma-Aldrich Ltd., UK). The bacteria were all Gram stained and subsequently observed microscopically to ensure they were Gram-negative bacilli. API testing, (Biomérieux, France) was used according to the manufacturer's instructions for final confirmation of bacterial identity.

2.3 P22 transduction of the disrupted region

The λ red recombinase used during the construction of the gene disrupted mutants is known to induce non-specific mutations throughout the genome. To remedy this problem the disrupted region containing the *aph* cassette was transduced back into SL1344 using P22 phage.

2.3.1 Production of the P22 phage

A 10 ml overnight culture of the strain containing the *aph* cassette was grown in LB broth at 37°C with shaking. This culture was diluted 1:100 (5 μ l in 5 ml) in fresh LB broth supplemented with 10 mM MgSO₄ and 5 mM CaCl₂ and grown at 37°C with aeration. After 30 minutes 5 μ l of P22 phage stock was added to the culture and re-incubated at 37°C with aeration overnight. 1 ml of chloroform was added to the bacteria and phage culture and this was vortexed hard for 20 seconds and then centrifuged at 2250 x *g* (Mistral 3000i, MSE) for 10 minutes at 4°C. The supernatant was then transferred to a glass universal container. 200 μ l of chloroform was added and vortexed briefly. The P22 phage stock was then stored at 4°C until needed.

2.3.2 P22 Transduction

A 10 ml culture of the recipient strain, SL1344 (L354), was grown overnight in LB broth at 37°C with aeration. The culture was centrifuged at 2250 x *g* (Mistral 3000i, MSE) at room temperature for 10 minutes. The supernatant was discarded and the pellet re-suspended in 1 ml 10 mM MgSO₄ and 5 mM CaCl₂. Six 100 μ l aliquots of the cell suspension were set up and into each aliquot a volume of phage lysate was added in a range from 0 μ l to 100 μ l. The transduction reactions were then incubated at 37°C for 15 minutes. 100 μ l of sodium citrate was added to chelate the divalent cations along with 1 ml of LB broth. This was incubated for 45 minutes at 37°C. To select for transductants, 100 μ l from each transduction reaction was spread onto LB agar supplemented with 50 μ g/ml

kanamycin and incubated at 37°C overnight. Putative transductants were confirmed by PCR (Table 2.5, PCR 2, 6 and 7).

2.4 Removal of the kanamycin resistance gene, *aph*, using the pCP20 plasmid

The kanamycin resistance gene, *aph*, was removed from the gene disrupted mutant L884 (*acrA::aph*) using the ampicillin resistant plasmid, pCP20 (Cherepanov and Wackernagel, 1995; Datsenko and Wanner, 2000) (Table 2.3). The plasmid contains the FLP gene whose product will remove the FRT-flanked antibiotic resistance cassette in the gene disrupted mutant. It is temperature sensitive such that it replicates at 30°C and is cured at 43°C (Datsenko and Wanner, 2000).

2.4.1 Treatment of L884 with pCP20

A single colony of the recipient strain, L884 (*acrA::aph*), was inoculated into 10 ml LB broth and grown overnight at 37°C with aeration. 4 ml of the overnight culture was added to 50 ml of pre-warmed LB broth (4% inoculum) and grown at 37°C with aeration for approximately 4 hours until mid-logarithmic phase ($OD_{600} = 0.6$). The cells were harvested by centrifugation at $2250 \times g$ for 10 min at 4°C (Mistral 3000i, MSE). The cell pellet was re-suspended in 50 ml of ice-cold 10% glycerol to wash. The washing process was repeated a further three times to make cells competent. After a final centrifugation the pellet of competent L884 cells was re-suspended in 300 µl of ice-cold 10% glycerol and left on ice. 5 µl of the isolated pCP20 plasmid was added to 45 µl of competent L884 cells and this was left on ice for 20 minutes. The cell/plasmid mixture was transferred to a chilled electroporation cuvette and electroporation was carried out at 2.5 kV. A negative control with no plasmid and a positive control with pUC19 were also included. After electroporation the cells were recovered in 1 ml of pre-warmed SOB broth. The recovery mixture was incubated at 30°C for one hour. 200 µl of the recovery mixture was spread onto LB agar plates containing 25 µg/ml ampicillin. The plates were incubated for 48 hours at 30°C and then inspected for candidate transformants.

2.4.2 Curing the plasmid

Candidate transformants were selected and inoculated into 10 ml of antibiotic free LB broth and grown overnight with aeration at 43°C to allow the plasmid to be cured. A loopful of this overnight culture was streaked onto antibiotic free LB agar and incubated at 37°C overnight. From this plate single colonies were selected and sub-cultured on three plates: antibiotic free LB agar, LB agar containing 25 µg/ml ampicillin to screen for loss of the pCP20 plasmid and a plate containing 25 µg/ml kanamycin to screen for loss of the *aph* gene.

2.4.3 Confirmation of removal of the *aph* gene

Candidate recombinants that were ampicillin and kanamycin susceptible were selected for further analysis. To confirm that the *aph* insertion had been removed from the *acrA* gene two PCRs were performed. The first PCR allowed assessment of the size of the *acrA* region (Table 2.5, PCR 2). In the wild-type strain (SL1344) this PCR would produce a product of 1528 bp while in L884 (*acrA::aph*) the product would be 2033 bp. If the *aph* cassette has been successfully removed then the PCR should produce a fragment of only 539 bp. A PCR using primers internal to the *aph* gene was performed to ascertain if it was still present (Table 2.5, PCR 8). The wild-type strain, SL1344, and a strain from which the *aph* gene has been removed should not produce a product in this reaction while a strain containing the *aph* gene (e.g. L884) would produce a product of 471 bp. The amplified products were analysed by running the samples on a 1% agarose gel, along with a DNA sizing ladder (Hyperladder I, Bioline, BIO-33025). This was electrophoresed at 100 volts for one hour and visualised on the 'Gene genius' image analyser (Syngene, UK).

2.5 Complementation of *acrA*

To complement the *acrA* mutants, wild-type *acrA* was cloned into the well characterised, low copy number vector pWKS30 (Wang and Kushner, 1991) (Table 2.3). This vector had been used previously

in this laboratory to complement $\Delta acrB$ and $\Delta tolC$ (Buckley *et al.*, 2006). It contains the ampicillin resistance marker and a multiple cloning site (MCS) containing, among others, HindIII and BamHI restriction sites.

2.5.1 Amplification and restriction digest of the wild-type *acrA* insert

Primers 648 and 649 were designed to amplify wild-type *acrA*, along with the region upstream of the gene so that the native promoter would be present to drive transcription of the cloned gene allowing approximately wild-type levels of *acrA* to be present in the complemented strain (Table 2.4). Restriction sites for HindIII and BamHI (AAGCTT and GGATCC, respectively) which are compatible with the MCS of the vector were added the 5' end of the primers along with two extra bases to allow the restriction enzymes to cut. Wild-type *acrA* was amplified by PCR using primers 648 and 649 (Table 2.4 and 2.5, PCR 9). Successful amplification was confirmed by running the samples on a 1% agarose gel along with a DNA ladder (Hyperladder I, Bioline, BIO-33025). This was electrophoresed at 100 V for one hour and visualised on the 'Gene genius' image analyser (Syngene, UK).

The amplified *acrA* fragment was digested with the restriction enzymes BamHI and HindIII (Both Promega Corporation, UK). 30 μ l of the PCR product was added to 5 μ l of the supplied multicore buffer, 5 μ l HindIII, 5 μ l BamHI, 1 μ l BSA and 4 μ l H₂O. This 50 μ l reaction was incubated at 37°C for one hour and inactivated at 80°C for 20 minutes.

2.5.2 Isolation and digestion of pWKS30 plasmid

The pWKS30 plasmid was isolated from *E. coli* strain I906. One colony of I906 was inoculated into LB broth and grown overnight at 37°C with aeration. The plasmid was harvested using the QIAprep® Miniprep kit (Qiagen, 27104) following the low copy number protocol to ensure maximum yield. The plasmid was then digested with HindIII and BamHI following the same protocol as for the PCR fragment digestion (Section 2.5.2). Successful plasmid isolation and digestion was confirmed by gel

electrophoresis on a 1% agarose gel which was visualised using the 'Gene genius' image analyser (Syngene, UK). The plasmid stock was stored at -20°C.

2.5.3 Ligation of *acrA* insert and pWKS30 plasmid

The digested plasmid and insert were ligated using the Bioline Quick Stick Ligase® (Bioline, 27027). 3 µl of digested *acrA* insert and 1 µl of digested plasmid were added to 10 µl of ddH₂O, 1 µl of Quick Stick DNA Ligase and 5 µl 4 x Quick Stick Buffer. The 20 µl reaction was mixed thoroughly by pipetting and incubated at room temperature for five minutes. The ligated product was loaded onto a 1% agarose gel alongside the digested insert, the digested plasmid and a DNA sizing ladder (Hyperladder 1, Bioline, BIO-33025) for comparison purposes. The gel was visualised on the 'Gene genius' image analyser (Syngene, UK). A successful ligation would produce a band of 6.9 Kb. It is also possible that bands of 1542 bp representing residual insert and of 5.4 Kb representing residual plasmid will also be present.

2.5.4 Transformation of DH5α cells with the ligated product

In the first instance the ligated product was electroporated into electro-competent *E. coli* DH5α cells (Invitrogen, UK) because high competence is guaranteed. 5 µl of the ligated plasmid was added to 45 µl of DH5α cells and this was left on ice for 20 minutes. The cell/plasmid mixture was transferred to a chilled electroporation cuvette and electroporation was carried out at 1.25 kV. A negative control containing no plasmid was also included. After electroporation the cells were recovered in 1 ml of room temperature SOB broth. The recovery mixture was incubated at 37°C for one hour. 200 µl of the recovery mixture was transferred onto LB agar plates containing 25 µg/ml ampicillin. The plates were incubated for 48 hours at 37°C and then inspected for candidate transformants.

All colonies which appeared on the selective media after 48 hours were sub-cultured onto individual agar plates containing 25 µg/ml ampicillin and incubated overnight at 37°C. A single colony from each

plate was inoculated into 10 ml of LB broth supplemented with 25 µg/ml ampicillin and incubated overnight at 37°C with aeration. Plasmid DNA was harvested using the QIAprep® Miniprep kit (Qiagen, 27104) following the low copy number protocol to ensure maximum yield. Successful plasmid isolation was confirmed by gel electrophoresis on a 1% agarose gel which was visualised using the 'Gene genius' image analyser (Syngene, UK).

To verify that the plasmid obtained from the candidate transformants was the complementation plasmid constructed, a restriction digest along with a PCR to check for fragment identity were performed. The plasmid was digested with HindIII and BamHI as previously described (Section 2.5.2) to see whether fragments of the predicted size were produced. The entire restriction digest reaction was loaded onto a 1% agarose gel. After electrophoresis, at 100 V for one hour, the gel was visualised using the 'Gene genius' image analyser (Syngene, UK). Fragments of 5.4 Kb and 1.5 Kb were expected, representing the plasmid and insert, respectively. The band representing the insert was excised and the DNA isolated using the QiaQuick Gel Extraction Kit (Qiagen, 28704). To confirm that this band contained the *acrA* insert a PCR was performed using primers upstream and downstream of *acrA* (Table 2.4, Primers 570 and 571 and Table 2.5, PCR 1). Successful amplification was confirmed by gel electrophoresis on a 1% agarose gel which was electrophoresed at 100 V for one hour and visualised using the 'Gene genius' image analyser (Syngene, UK). The production of an amplicon of 1528 bp would indicate that the plasmid in the candidate transformant contained the *acrA* insert.

2.5.5 Transformation of L884 (*acrA::aph*) with pWKS30*acrA*

The transformed *E. coli* 1750 strain containing the pWKS30*acrA* plasmid was grown overnight in LB broth containing 25 µg/ml ampicillin at 37°C with aeration. The plasmid was harvested using the QIAprep® Miniprep kit (Qiagen, 27104) following the low copy number protocol to ensure maximum yield. Successful plasmid isolation was confirmed by gel electrophoresis on a 1% agarose gel and visualization using the 'Gene genius' image analyser (Syngene, UK).

A single colony of the recipient strain, L884 (*acrA::aph*), was inoculated into 10 ml LB broth and grown overnight at 37°C with aeration. 4 ml of the overnight culture was added to 50 ml of pre-warmed LB broth (4% inoculum) and grown at 37°C with aeration for approximately 4 hours until mid-logarithmic phase ($OD_{600} = 0.6$). The cells were harvested by centrifugation at $2250 \times g$ for 10 minutes at 4°C (Mistral 3000i, MSE). The cell pellet was re-suspended in 50 ml of ice-cold 10% glycerol to wash. The washing process was repeated a further three times. After a final centrifugation the pellet of competent L884 cells was re-suspended in 300 µl of ice-cold 10% glycerol and left on ice.

5 µl of plasmid DNA was added to 45 µl of the competent L884 cells and left on ice for 20 minutes. The cell/plasmid mixture was transferred to a chilled electroporation cuvette and electroporation was carried out at 2.5 kV. A negative control containing no plasmid was also included. After electroporation the cells were recovered in 1 ml of room temperature SOB broth. The recovery mixture was incubated at 37°C for one hour. 200 µl of the recovery mixture was transferred onto LB agar plates containing 25 µg/ml ampicillin. The plates were incubated for 48 hours at 37°C and then inspected for candidate transformants.

All colonies which appeared on the selective media after 48 hours were sub-cultured onto individual agar plates containing 25 µg/ml ampicillin and incubated overnight at 37°C. A single colony from each plate was inoculated into 10 ml of LB broth supplemented with 25 µg/ml ampicillin and incubated overnight at 37°C with aeration. Plasmid DNA was harvested using the QIAprep® Miniprep kit (Qiagen, 27104). Successful plasmid isolation was confirmed by gel electrophoresis on a 1% agarose gel and visualised using the 'Gene genius' image analyser (Syngene, UK). Identity of the plasmid was confirmed by performing a restriction digest and PCR of the insert to check for fragment identity as in Section 2.6.7.

2.6. Measuring transcription and translation of *acrA*/AcrA and *acrB*/AcrB

Reverse transcriptase (RT) PCR was used to measure the transcription of the *acrA* and *acrB* genes and Western Blotting was used to measure the level of AcrA and AcrB proteins.

2.6.1 Determination of *acrA* and *acrB* transcription

2.6.1.1 RNA extraction

To give three biological and three technical replicates of each strain, on three separate occasions single colonies of the strains to be tested were each inoculated into 10 ml LB broths and incubated overnight at 37°C with aeration. 1 ml of overnight culture was inoculated into 24 ml of pre-warmed minimal media (Teknova, US) and incubated at 37°C with aeration until mid-logarithmic phase (OD_{600} of 0.6). 5 ml of culture was added to 1 ml of a 95% ethanol, 5% phenol mixture and left on ice for 30 minutes. The samples were centrifuged at $2250 \times g$ (Mistral 3000i, MSE), 4°C for 10 minutes and the supernatant discarded. The pellets were re-suspended in 100 μ l of TE buffer containing 50 mg/ml lysozyme and incubated at room temperature for 5 minutes. After lysozyme treatment the Promega SV Total RNA Purification Kit was used (Promega Corporation, UK, Z3100), according to manufacturer's instructions. The isolated RNA was loaded onto a 1% agarose gel along with a DNA sizing ladder (Hyperladder I, Bioline, BIO-33025) and electrophoresed at 100 volts for one hour and visualised on the 'Gene genius' image analyser (Syngene, UK) to see whether RNA was visible. A PCR was performed to see whether any residual DNA was present in the sample (Table 2.5, PCR 4). Any residual DNA was removed by digestion with Promega RNase free DNase (Promega Corporation, UK, M6101) according to the manufacturer's instructions.

2.6.1.2 cDNA synthesis

cDNA was synthesised using Invitrogen Superscript III Reverse Transcriptase (Invitrogen, UK). For first strand DNA synthesis 1 µg of RNA was added to 1 µl of 50 ng/µl Random Primers, 1 µl dNTP mix and this was made up to 10 µl with RNase free sterile water in nuclease free microcentrifuge tubes. The reaction was incubated at 65°C for five minutes and then on ice for one minute. 10 µl of the RNA mix was added to 10 µl of cDNA synthesis mix consisting of 2 µl of 5 x first strand buffer, 4 µl of 25 mM MgCl₂, 2 µl 0.1 M DTT, 1 µl 40 U/µl RNase Out and 1 µl of 100 U/µl Superscript III reverse transcriptase. This was incubated at 25°C for 10 minutes and then at 50°C for 50 minutes. The reaction was terminated at 85°C for five minutes. The cDNA was stored at -20°C.

2.6.1.3 RT-PCR

PCRs for *acrA*, *acrB* and 16s (Table 2.5, PCRs 3, 4, and 5) were set up using the cDNA as a template. For the 16s PCR cDNA was used at a 1:1000 dilution while for both *acrA* and *acrB* PCRs the cDNA was used at a dilution of 1:5 (Eaves *et al.*, 2004). The products of all PCR reactions were loaded onto a 1% agarose gel along with a DNA sizing ladder (Hyperladder I, Bioline, BIO-33025) and electrophoresed at 100 V for approximately one hour. The gel was visualised on the 'Gene genius' image analyser (Syngene, UK) and inspected for presence or absence of amplified products. PCR products were then loaded onto a dHPLC column (Transgenomic Ltd, UK).

2.6.1.4 PCR product quantification using Denaturing High Pressure Liquid Chromatography (DHPLC)

The PCR products were quantified using DHPLC and the WAVE DNA fragment analysis system (Transgenomic Inc.). The method used was as published by Eaves *et al* (2004). This system separates fragments by size using heat (50°C) and ion-pair chromatography. PCR products were loaded onto the machine in open topped 0.2 ml tubes and from here 15 µl of each PCR product was loaded onto the column (DNASepCartridge, Transgenomic Inc.) with 55% buffer A (0.1M

triethylammoniumacetate, TEAA) and 45% buffer B (0.1M TEAA in 25% acetonitrile). A buffer gradient from 50% buffer B to 65% buffer B was created over 15 minutes with a flow rate of approximately 0.9ml/min to allow separation of the products. The column was then washed with 100% buffer B for 30 seconds. The elution profiles of each sample were quantified according to their peak areas with the NAVIGATOR software (version 1.4) (Transgenomic Inc.).

Average transcription for the genes of interest were calculated from each cDNA synthesis reaction. The level of 16s rRNA transcription has previously been shown to be invariant in the conditions (Eaves *et al.*, 2004; Bailey, 2007). Therefore, the level of 16s transcription was used to normalise the transcription level of each test gene as previously described (Eaves *et al.*, 2004; Bailey, 2007). After normalisation, the mean and standard deviation of transcription levels were calculated and converted to fold change compared to SL1344. Student's *t*-tests were calculated to compare transcription of genes relative to SL1344.

2.6.2 Western Blotting

2.6.2.1 Isolation of membrane proteins

Single colonies of the bacterial strains to be tested were each inoculated into 10 ml LB broths and incubated overnight at 37°C with aeration. 1 ml of overnight culture was inoculated into 50 ml of pre-warmed LB broth and incubated at 37°C with aeration until mid-logarithmic phase (OD_{600} of 0.6). Cultures were centrifuged at 2250 x *g* for 15 minutes at 4°C and the cells re-suspended in 1 ml of 10 mM Tris-EDTA buffer, pH 8.5 (Fluka, 93283). Lysozyme was added to a final concentration of 100 µg/ml and incubated on ice for 30 minutes. Cells were disrupted by sonication on ice; three pulses of 30 seconds, with 30 seconds rest in between. Unbroken cells were removed by centrifugation at 2250 x *g* and the supernatant was centrifuged at 100,000 x *g* for 1 hour at 4°C. The

pellet, containing the total membrane proteins, was re-suspended in 200 µl of 10 mM Tris-EDTA buffer, pH 8.5.

The protein content of each membrane preparation was determined using a Bradford assay. 20 µl of each membrane preparation was added to 980 µl of Bradford reagent (Sigma Aldrich, UK, B6916) and the OD₅₉₅ was measured. 1 ml of Bradford agent alone was used as the blank. Protein content was calculated by comparison to a curve produced using Bradford reagent and known protein standards.

2.6.2.2 Western blotting

The volume of each membrane protein preparation required to give 3 µg of protein in each lane was calculated, mixed with NuPAGE® LDS sample buffer (Invitrogen, NP0007) and loaded onto a NuPAGE® 10% Bis-Tris gel (Invitrogen, NP0303BOX) along with a molecular weight marker (Magic Mark XP Western Standard, Invitrogen, LC5602). This was electrophoresed at 4°C in MOPs 1 x SDS Running Buffer (Invitrogen, NP0001) (50 ml of 20 x MOPS SDS running buffer in 950 ml water) at ~100v until the dye front reached the bottom of the gel. For each new set of protein preparations the first SDS-PAGE gel was stained with Safestain® (Invitrogen, LC6060), a coomassie blue like stain, to ensure that the protein preps were adequate and that an equivalent amount of protein was present in each well.

For subsequent immunoblotting the proteins were transferred to a PVDF membrane (GE Healthcare, RPN1416LFP) by electrophoresis at 50v for three hours at 4°C in an electro-blotting unit (Scie-Plas, UK, EB10) filled with transfer buffer. Transfer buffer was made by adding 100 ml of methanol to 5.8 g Glycine (Fluka, 50046) and 11.6 g Trizma base (Sigma, T1503). This was then made up to 2 L and the pH adjusted to 8.3 with HCl. After transfer the membrane was washed twice for five minutes each with ~40 ml of 1 x Tris-Buffered Saline (TBS) with vigorous shaking. 10 x TBS was prepared by adding 24.2 g of Trizma base and 80 g of NaCl to 800 ml of distilled water. The pH was adjusted to 7.6 with

HCl and the volume was made up to 1 L with distilled water. This solution was diluted 1:10 before use. The membrane was blocked by incubation with ~40ml blocking buffer (1 x TBS with 0.1% Tween-20 (Sigma, P5927) and 5% w/v skimmed milk powder (Sigma 70166)) for one hour at room temperature and then washed three times for 5 minutes with TBS/T (1 x TBS with 0.1% Tween-20). The blocked membrane was then washed three times for 5 minutes with TBS/T and incubated overnight at 4°C with either AcrA, AcrB or TolC primary antibody diluted 1:60,000 with blocking buffer (kindly provided by Helen Zgurskaya, Elena Tikhonova and Vassilis Koronakis). The membrane was then washed three times for five minutes with ~40 ml of TBS/T with vigorous shaking and then incubated for a further hour with Anti-Rabbit IgG, HRP-linked secondary antibody raised in donkey (GE healthcare, NA934V). After a further three washes the blot was flooded with mixed ECL western blotting detection system (GE Healthcare, RPN2132) on the protein side for 5 minutes. The excess detection fluid was drained from the membrane and the membrane placed protein side down on saran wrap which is wrapped around the blot. To visualise the blot the wrapped membrane was exposed to a sheet of chemi-luminescence autoradiography film (Amersham Hyperfilm ECL 28906836) for between 2 and 30 minutes. The film was developed immediately using Kodak developer and fixer (Kodak, 190 0943 and 190 1875, respectively).

2.7 Phenotypic Characterisation of *S. Typhimurium* *acrA::aph*

To determine the effect of inactivating the *acrA* gene the characteristics of the *acrA* mutant strains were compared to that of the wild-type (SL1344) and other efflux mutants L109 (*tolC::aph*) and L110 (*acrB::aph*) in a series of phenotypic tests.

2.7.1 Bacterial growth kinetics

Each strain was grown overnight at 37°C in LB broth containing appropriate antibiotics where needed. The overnight cultures were diluted 1:100 into LB broth. 100µl of this diluted culture was

added to each well of a 96 well plate. Optical density at 600nm was measured every ten minutes for 12 hours in the BMG FLUOstar optima (BMG, UK). All growth experiments were conducted at 37°C. Each experiment was repeated three times and each repeat included eight technical repeats of each strain. The FLUOstar is sensitive to an OD600 of between 0.0 and 4.0 and reproducibility is ± 0.010 for the OD range of 0.0-2.0 (www.bmglabtech.com).

2.7.2 Determination of the Minimum Inhibitory Concentration (MIC) of antibiotics

The MIC of a series of antibiotics, dyes and detergents (Table 2.2) were determined using the agar doubling dilution method as described by the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2006). All antibiotics used in this study are shown in Table 2.2. Fresh stock solutions of each antibiotic were made on the day of use. Appropriate volumes of the antimicrobial stock were dispensed into sterile plastic universal containers to which 20 ml of cooled, molten, Iso-sensitest agar (Oxoid, UK) was added aseptically with a peristaltic pump dispenser (Jencons Scientific Ltd., UK). The antimicrobial and agar were mixed and poured into labelled tri-vented petri dishes and allowed to set. The plates were dried for approximately 15 minutes at 50°C in an oven. Overnight cultures of each bacterial strain to be tested were diluted 1:100 to give approximately 10^7 CFU/ml. Each agar plate was inoculated with 1 μ l of diluted culture giving approximately 10^4 CFU/spot. Inoculation was carried out using a 21 pin multipoint inoculator (AQS manufacturing, UK). The plates were incubated overnight at 37°C. The MIC is defined by BSAC as the lowest concentration of a drug that that will inhibit the visible growth of an organism after overnight incubation (Andrews, 2006). MICs were determined as the modal value from at least three independent experiments.

2.7.3 Accumulation of Hoechst 33342 (Bis-benzamide)

Hoechst, H33342 (bis-benzamide) fluoresces when bound to DNA and therefore its accumulation can be used to assess the relative level of active efflux in the mutants (Webber *et al.*, 2008; Webber and

Coldham, In Press). To give three biological replicates of each strain, on three separate occasions single colonies of the strains to be tested were each inoculated into 10 ml LB broth and incubated overnight at 37°C with aeration. 1 ml of overnight culture was inoculated into 10 ml of pre-warmed LB broth and incubated at 37°C with aeration until mid-logarithmic phase (OD_{600} of 0.6). Cells were harvested by centrifugation at 2250 x *g* and re-suspended in Phosphate Buffered Saline (PBS) to an optical density of 0.1 at a wavelength of 600 nm. 200 µl of diluted culture was added to each well of a black flat bottomed 96 well plate (Greiner CN 655076). A 25 µM Hoechst H33342 stock was prepared and loaded into the FLUOstar OPTIMA (BMG labtech) for injection after an initial reading, to a final concentration of 2.5 µM. Fluorescence intensity was measured over 30 minutes at excitation and emission wavelengths of 350 and 461 nm, respectively in a FLUOstar OPTIMA (BMG labtech). Student's *t*-tests were performed to compare the accumulation of each strain to that of the parental wild-type strain, SL1344; *P* values <0.05 were taken as significant.

2.7.4 Electron microscopy of SL1344 and strains lacking AcrA, AcrB and TolC

The strains SL1344, L109, L110 and L884 were grown and given to Bill Cooley at the veterinary laboratories agency, Weybridge, Surrey for transmission electron microscopy studies. Briefly, samples were fixed using 2.5% glutaraldehyde in phosphate buffer and then 1% osmium tetroxide. Samples were dehydrated through a graded ethanol series then embedded in epoxy resin. Sections were cut at 70-90 nm thickness and stained with uranyl acetate and Reynolds lead citrate.

2.8 Investigating the Role of AcrAB-TolC in Adhesion and Invasion

The ability of the *acrA* mutant strains to adhere and invade to two tissue culture cell lines *in vitro* was determined and compared to that of the wild-type strain (SL1344) and two other efflux mutants (L109 and L110). The cell lines used were human embryonic intestine cells (INT-407) and mouse macrophages (RAW 264.7) as used previously by this team (Buckley *et al.*, 2006). The protocols used

for the adhesion and invasion assays were based upon those described by Dibb-Fuller and colleagues (Dibb-Fuller *et al.*, 1999). Each assay was repeated a minimum of three times and each repeat contained four technical repeats. Both the INT-407 and RAW 264.7 cell lines were kindly supplied by R. La Ragione at the Veterinary Laboratories Agency (VLA), Addlestone, Surrey.

2.8.1 Tissue culture

Monolayers of each cell type were grown in 250ml tissue culture flasks (BD Falcon™, 353136) in complete tissue culture medium. INT-407 cells required Minimum Essential Medium (MEM) (Sigma-Aldrich, UK, M2279) while RAW cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, UK, D5671). The complete tissue culture medium contained the appropriate medium for the cell type supplemented with 10% heat inactivated foetal calf serum (Invitrogen, UK, 10108-165), 1% non-essential amino acids (NEAA) (Sigma, UK, M7145), 1% L-glutamine (Sigma, UK, G7513) and 50µg/ml gentamicin (Sigma, UK, G1397). For the assays monolayers of each cell line were prepared in 24 well plates (Falcon). Each well was seeded with approximately 10^5 cells and incubated for 48 hours at 37°C and 5% CO₂. Before the assays were performed each well was washed with 1 ml pre-warmed Hanks Balanced Salt Solution (HBSS) (Sigma, UK, H9269) by pipetting 1 ml into each well and then removing it. This was done three times for each well to remove residual medium and antibiotic.

2.8.2 Preparation of bacterial strains for infection assays

All the bacterial strains were grown overnight at 37°C in LB broth containing appropriate antibiotics where necessary. The cells were harvested from the culture by centrifugation at 2250 x *g* for 10 minutes at 4°C in a Mistral 3000i. The supernatant was discarded and the cells were re-suspended in 10ml of sterile PBS to wash and re-centrifuged. The supernatant was once more discarded and the cells were re-suspended in approximately 10 ml of sterile PBS and adjusted to an optical density of

1.23 at 540 nm with further PBS (approximately 1×10^7 CFU/ml). The bacterial cultures were then diluted 1:20 in appropriate inoculation medium and viable counts were performed on this diluted bacterial culture. Inoculation media consisted of the appropriate medium for the intended cell type (MEM or DMEM) supplemented with 1% L-glutamine (Sigma, UK) and 1% NEAA (Sigma, UK).

2.8.3 The Association Assay

To quantify the number of bacteria which associated with the eukaryotic cell monolayer 1 ml of the diluted bacterial suspension was inoculated into each well and incubated for two hours at 37°C and 5% CO₂ as described previously (Dibb-Fuller *et al.*, 1999; Buckley *et al.*, 2006). The supernatant was then removed and the monolayers were washed six times with 1 ml of pre-warmed HBSS to remove all non-adherent bacteria. The monolayers were disrupted by adding 1 ml of sterile 1% Triton-X 100 and a magnetic flea to each well. The plate was put onto a magnetic stirrer for 10 minutes. A series of 1:10 dilutions were made from each well of the tissue culture plate and three 20 µl drops of each dilution were plated onto LB agar (Miles *et al.*, 1938). The agar plates were incubated overnight at 37°C. The colonies from those dilutions that gave separate colonies were counted and used to determine the number of colony forming units (CFU) per millilitre.

2.8.4 The Invasion Assay

To determine the number of bacteria which invaded the eukaryotic cell monolayer 1 ml of bacterial suspension diluted 1:20 in inoculation media was added to each well and incubated for two hours at 37°C and 5% CO₂. The supernatant was then removed and the monolayers were washed three times with 1 ml of pre-warmed HBSS to remove all non-adherent bacteria. Two millilitres of media containing 100 µg/ml of gentamicin was added to each well and the plate was incubated for a further two hours at 37°C and 5% CO₂. This step kills all external bacteria without damaging or entering the eukaryotic cells, so leaving the internal bacteria unharmed. The antibiotic containing media was then

removed and the monolayers were washed six times with 1 ml of pre-warmed HBSS. The monolayers were disrupted by adding 1 ml of 1% Triton-X 100 and a magnetic flea to each well. The plate was put onto a magnetic stirrer for 10 minutes. A series of 1:10 dilutions were made from each well of the tissue culture plate and three 20 μ l drops from each dilution were plated onto LB agar (Miles *et al.*, 1938). The agar plates were incubated overnight at 37°C. The colonies from those dilutions that gave separate colonies were counted and used to determine the number of colony forming units (CFU) per millilitre.

2.8.5 Statistical Analyses

2.8.5.1 Mean and standard deviation

The mean and standard deviation CFU/ml was calculated for association and invasion for each biological replicate. Each experiment was performed a minimum of three times and the overall mean CFU/ml for each strain was calculated as the mean of the values for each biological replicate. These values were plotted on a graph in the Excel software (Microsoft, 2007) with error bars \pm one standard deviation.

2.8.5.2 Determining the level of Adhesion

The level of adhesion was determined by subtracting the mean CFU/ml for invasion from the mean CFU/ml for association. These values were plotted on a graph in the Excel software (Microsoft, 2007) with error bars \pm one standard deviation.

2.8.5.3 Student's *t*-Test

A Student's *t*-test was performed to compare the adhesion and invasion values of each strain to wild-type values. The test was performed using the *t*-test function of the Excel software (Microsoft, 2007). *P* values of ≤ 0.05 were deemed to be statistically significant.

2.8.5.4 Calculating the confidence interval for the association level of SL1344

The confidence interval at the 0.05 (95%) level of significance was calculated from the mean and standard deviation of all values obtained for association of SL1344 with INT-407 cells.

95% confidence interval = sample mean \pm (t-crit x SE of the mean),

Where the SE of the mean = $\frac{s}{\sqrt{n}}$ ()

Data from all future assays was only accepted if the SL1344 association values fell within the calculated range.

2.8.5.5 Multiplicity of Exposure

For each assay the multiplicity of exposure (MOE) was calculated. This is a measure of the number of bacterial cells present compared to the number of eukaryotic cells and is calculated by dividing the viable counts for each bacterial strain by the estimated number of eukaryotic cells per well. The number of eukaryotic cells per well was estimated by removing the tissue culture cells from monolayers in 10 wells by trypsinisation. The cells suspended in trypsin were added to 10 ml of complete tissue culture medium and centrifuged at 250 x g for 5 minutes (Mistral 3000i, MSE). The cells were then re-suspended in a known volume of media and counted using a haemocytometer. The estimated number of cells per well could then be calculated. The bacterial viable counts were performed by plating out serial dilutions of the diluted bacterial suspensions.

To check that the number of viable eukaryotic cells did not change during the assay cell counts using trypan blue were performed before and after infection. The number of viable eukaryotic cells per well was estimated by removing the tissue culture cells from monolayers in 4 wells by trypsinisation. The cells suspended in trypsin were added to 10 ml of complete tissue culture medium and centrifuged at 250 x g for 5 minutes (Mistral 3000i, MSE). The cells were re-suspended in a known

volume of complete media and trypan blue added for 5 minutes before being counted using a haemocytometer. Dead cells take up the stain so appear blue while viable cells do not and are colourless.

2.9 Competition assays

10 ml bacterial cultures of the two strains to be tested were grown overnight at 37°C. 1ml of each culture was diluted in 5 ml PBS and the optical density at a wavelength of 600 nm was measured. The OD of the culture with the highest starting value was adjusted to be equal to that of the other culture by addition of further PBS. 20 µl was taken from each culture, serially diluted and pipetted out using the method described by Miles and Misra (Miles *et al.*, 1938). Equal volumes of the two solutions were then combined, and another 20 µl sample was taken, serially diluted and pipetted in the same manner. The mixed culture solution was centrifuged at 2250 x *g* for 10 minutes at 4°C. As described in section 2.8.2 the supernatant was discarded and the pellet re-suspended in PBS. The OD of this re-suspended pellet was then adjusted to 1.23.

Association and invasion assays were performed as described in section 2.8.3 and 2.8.4. Each strain was tested alone as well as in competition. At the end of the assay the LB agar plates were incubated overnight at 37°C as normal. Replica plating onto LB agar containing 50 µg/ml of kanamycin allowed SL1344 and the kanamycin resistant mutant (containing the *aph* gene) to be distinguished. The colonies from those dilutions that gave separate colonies were counted and used to determine the number of colony forming units (CFU) per millilitre.

2.10 Does AcrAB-TolC export a molecule required for invasiveness?

Experiments were performed to ascertain whether the invasiveness of L109 (*tolC::aph*) *in vitro* could be restored by the addition of culture supernatant from INT-407 cells infected with wild-type *Salmonella*, SL1344.

2.10.1 The effect of addition of culture supernatant, INT-407 conditioned media or SL1344 conditioned media on the invasive abilities of mutants

The adhesion and invasion assays were performed as described in sections 2.8.3 - 2.8.5. Bacterial suspensions were prepared as described in section 2.8.2 except that the bacteria were diluted 1:20 in either inoculation media (MEM supplemented with 1% L-glutamine and 1% NEAA), culture supernatant from INT-407 cells infected with SL1344 (section 2.10.11), INT-407 conditioned media (section 2.10.12) or SL1344 conditioned media (section 2.10.13).

2.10.1.1 Generation of culture supernatant from SL1344 infected INT-407 cell monolayers

Each well of a 24 well plate of confluent INT-407 monolayers was washed three times with 1 ml of pre-warmed HBSS (Sigma, UK, H9269). An overnight broth culture of SL1344 was diluted in inoculation media as described (Section 2.8.2) and 1 ml of diluted culture was added to each well. The plates were then incubated for 30 minutes, two hours or 18 hours at 37°C and 5% CO₂. After incubation the supernatant was collected and filter sterilised using either a single use Millex® GP 0.45µm filter unit or a single use low protein binding 0.45 µm filter (Millex®HV, Durapore® PVDF membrane). The sterile culture supernatant was diluted 1:2 and 1:20 in inoculation media and then used to dilute the bacterial suspension 1:20. For the control, without supernatant, the bacterial suspension was diluted 1:20 in inoculation media alone.

2.10.1.2 Generation of INT-407 conditioned media

Each well of a 24 well plate of confluent INT-407 monolayers was washed three times with 1 ml of pre-warmed HBSS (Sigma, UK, H9269). 1 ml of sterile inoculation media was added to each monolayer and incubated for 30 minutes at 37°C and 5% CO₂. This media was then removed and filter sterilised using a single use low protein binding 0.45 µm filter (Millex®HV, Durapore® PVDF membrane). The sterile media was diluted 1:2 in inoculation media and then used to dilute the

bacterial suspension 1:20 (Section 2.8.2). For the control, without INT conditioned media, the bacterial suspension was diluted 1:20 in inoculation media alone.

2.10.1.3 Generation of SL1344 and L109 (*tolC::aph*) conditioned media

A 10 ml overnight LB broth culture of SL1344 was centrifuged at 2250 x *g* for 10 minutes at 4°C (Mistral 3000i, MSE) to pellet the bacteria and then filter sterilised using a single use low protein binding 0.45 µm filter (Millex®HV, Durapore® PVDF membrane). The SL1344 conditioned media was diluted 1:2 in inoculation media and then used to dilute the bacterial suspension 1:20 (Section 2.8.2). For the control, without SL1344 conditioned media, the bacterial suspension was diluted 1:20 in inoculation media alone. As another control, the bacterial suspension was also diluted in fresh LB media mixed 1:2 with inoculation media to ensure addition of LB broth had no effect.

2.10.1.4 Generation of boiled and proteinase K treated bacterially conditioned medium

The bacterially conditioned medium was boiled to determine whether this would affect its ability to restore the invasive ability of efflux mutants (L109, L110 or L884). The bacterially conditioned media was generated in exactly the same way as in section 2.10.1.3 but before dilution the media was put into a boiling water bath for 10 minutes. After being left to cool, the media was diluted 1:2 in inoculation media and used in the infection assays as described.

To determine whether the active component of the SL1344 conditioned media was proteinaceous, the bacterially conditioned media, which was generated as described in section 2.10.1.3, was treated with proteinase K. Proteinase K (Promega) was added to the conditioned media to a final concentration of 100 µg/ml and at 37 °C for one hour. The proteinase K was inactivated using by addition of EGTA to a final concentration of 10 mM. After inactivation, the proteinase K treated media was diluted 1:2 in inoculation media and used in the infection assays as described.

2.11 Imaging of infected INT-407 cells

2.11.1 Constructing Green Florescent Protein (GFP) marked strains

The pUA66*pacpP* plasmid was a gift from Neil Burton at the University of Birmingham and encodes a constitutively transcribed *gfp* gene. Briefly, the parent vector was pUA66 into which the *pacpP* promoter was cloned upstream of GFP*mut2*. This vector also contains the *aph* gene coding for kanamycin resistance.

A single colony of the recipient strain (SL1344, L109, L110 and L884) was inoculated into 10 ml LB broth and grown overnight at 37°C with aeration. 4 ml of the overnight culture was added to 50 ml of pre-warmed LB broth (4% inoculum) and grown at 37°C with aeration until mid-logarithmic phase ($OD_{600} = 0.6$). The cells were harvested by centrifugation at 2250 x *g* for 10 minutes at 4°C (Mistral 3000*i*, MSE). The cell pellet was re-suspended in 50 ml of ice-cold 10% glycerol to wash. The washing process was repeated a further three times. After a final centrifugation the pellet of competent cells was re-suspended in 300 µl of ice-cold 10% glycerol and left on ice.

5 µl of plasmid DNA was added to 45 µl of the competent recipient strain and left on ice for 20 minutes. The cell/plasmid mixture was transferred to a chilled electroporation cuvette and electroporation was carried out at 2.5 kV. A negative control containing no plasmid was also included. After electroporation the cells were recovered in 1 ml of room temperature SOB broth at 37°C for one hour. 200 µl of the recovery mixture was transferred onto LB agar plates containing 50 µg/ml kanamycin. The plates were incubated for 48 hours at 37°C and then inspected for candidate transformants.

All colonies which appeared on the selective media after 48 hours were sub-cultured onto individual agar plates containing 25 µg/ml kanamycin and incubated overnight at 37°C. A single colony from each plate was inoculated into 10 ml of LB broth supplemented with 25 µg/ml kanamycin and

incubated overnight at 37°C with aeration. Plasmid DNA was harvested using the QIAprep® Miniprep kit (Qiagen, 27104). Successful plasmid isolation was confirmed by electrophoresis of a 1% agarose gel and visualised using the 'Gene genius' image analyser (Syngene, UK).

To confirm successful transformation of the strains with pUA66pacpP the fluorescence of putative transformants was measured. GFP has a major excitation peak at a wavelength of 395 nm with emission at 509 nm.

Cultures of SL1344 and the putative transformants were grown in LB broth overnight at 37°C. The cultures were diluted 1:100 in sterile PBS (Invitrogen) and 3 ml of the diluted culture was loaded into a clean glass cuvette. The cuvette was loaded into the Perkin Elmer Fluorescence spectrophotometer (Perkin Elmer, LS 45). The excitation was set at 395 nm and a scan of emission wavelengths was run. As a control the emission of PBS alone and SL1344 in PBS was also measured.

2.11.2 Preparing infection plates, staining and imaging

For INT-407 infections a sterile circular glass slide was placed into each well of a 24 well plate (Falcon). INT-407 cells were then seeded into the 24 well tissue culture plate, on top of the glass slides, as described in section 2.8.1. For Caco2 infections, the cells were maintained in DMEM with 20% serum as described for INT-407 and RAW cells in section 2.8.1. For infection assays the cells were seeded onto transwell polyester membranes in 6 well plates (Costar). The media was replaced every two to three days for 12-14 days until infection. The INT-407 cells were infected as described in section 2.8.3. For the Caco2 cells the media was removed from the top portion of the transwell system and replaced with 1.5 ml of diluted bacterial culture, prepared as described in section 2.8.2. All infected cells were incubated at 37°C, 5% CO₂ for 2 hours. After this all spent media was removed and the infected eukaryotic cells washed with HBSS as described previously. For the INT cells, 1 ml of 1% formalin was added to each well, for the Caco2 cells 3 ml of 1% formalin was added to the

bottom chamber of the transwell system and 1.5 ml was added to the top compartment. Fixed plates were kept at 4°C until needed.

Staining and imaging of the fixed infected plates was kindly performed by Robert Shaw (University of Birmingham). Briefly, the plates were counterstained with the F-actin specific molecule phalloidin conjugated to the Rhodamine dye, so the eukaryotic cells stained red. Confocal z-stack images of the plates were then taken in the xzy (vertical) and xzy (transverse) planes.

2.11.3 Test to see whether the strains aggregate in broth culture

Strains were grown overnight in LB broth at 37°C with shaking at 150 rpm. Cultures were then left on the bench for eight hours to settle. At the beginning of the experiment and every hour thereafter 100 µl of culture was taken from the very surface of the culture. This was diluted in 900 µl of fresh broth and the optical density at 600 nm was recorded.

Chapter Three

Construction and Characterisation of Mutants Lacking AcrA

Chapter 3. Construction and Characterisation of mutants lacking AcrA

3.1 Background

It has previously been shown that inactivation of either *acrB* or *tolC* of *Salmonella* Typhimurium results hypersusceptibility to a range of antibiotics, dyes and detergents and an impaired ability to cause infection (Eaves *et al.*, 2004; Buckley *et al.*, 2006; Nishino *et al.*, 2006).

3.2 Aims and hypothesis

The initial aim was to inactivate the final component of the AcrAB-TolC efflux system, the periplasmic adaptor, *acrA*, while retaining transcription and translation of *acrB*/AcrB and *tolC*/TolC, respectively. The hypothesis was that inactivation of *acrA* would also lead to hyper-susceptibility to a range of antimicrobials by impairing pump function. In order to test this hypothesis, the mutant phenotype was characterised by studying the mutant growth kinetics, determining the minimum inhibitory concentrations (MIC) of a range of antimicrobials and measuring efflux activity in the mutant strain.

3.3 Disruption of *acrA*

The *acrA* gene was inactivated using the method published by Datsenko and Wanner in 2000 (Datsenko and Wanner, 2000). This method allows disruption of chromosomal genes by homologous recombination with PCR products containing the *aph* gene whose product provides kanamycin resistance. This relies on the plasmid encoded λ red recombination system. In the first instance the *aph* gene was amplified by PCR using primers that also have homology to the gene of interest. The recipient strain, containing the pKD46 plasmid, is

transformed with the PCR construct and homologous recombination follows, mediated by the λ red recombinase system, to leave a mutant with the chromosomal gene of interest disrupted by the kanamycin resistance gene, *aph*.

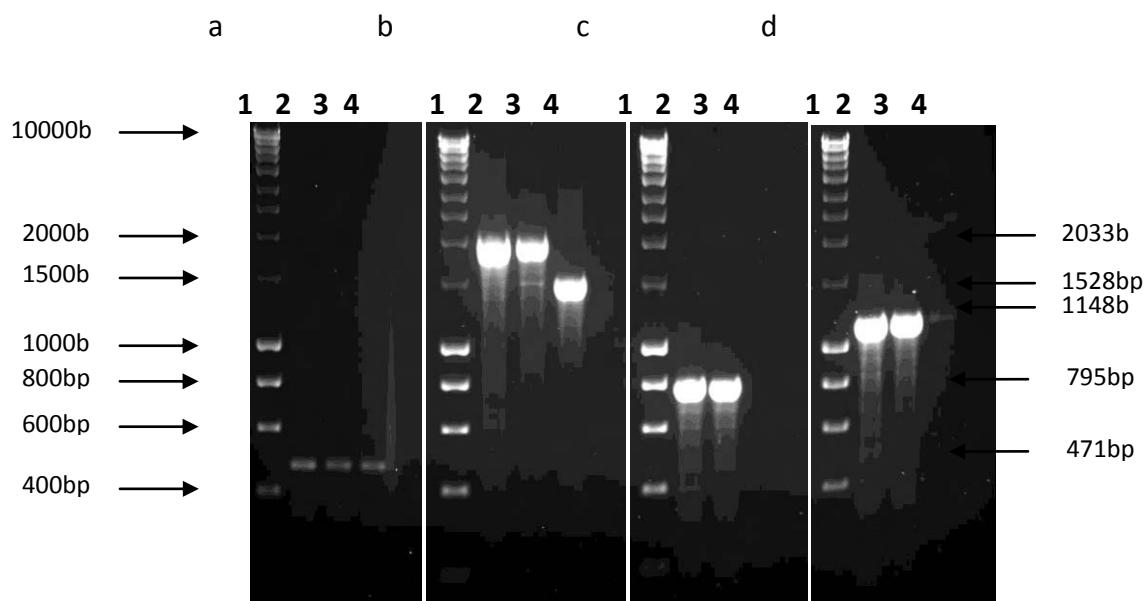
3.3.1 Verification of Gene Disruption

Following electroporation with the PCR amplified *aph* gene, potential recombinants were selected on LB agar with 50 μ g/ml kanamycin. After 48 hours two putative recombinants were present on the selective agar both of which were checked for the insertion of the kanamycin resistance gene (*aph*) by four PCR reactions (Table 2.5, PCR numbers 8, 2, 6 and 7) (Primer positions shown in Appendix 1, Figure 1). Following the PCR reactions, the amplicons were electrophoresed and visualised on the Gene genius [™] image analyser. Both candidates produced amplicons of the size predicted for an *acrA* disrupted mutant in all four of the PCR reactions (Figure 3.1).

To confirm insertion of the *aph* gene in the correct location, the PCR amplicons obtained using a primer that annealed in the *acrA* gene and one that annealed in the *aph* gene were sequenced (Table 2.5, PCRs 6 and 7). The sequence was aligned with the predicted sequence for an *acrA::aph* mutant (Appendix 1). The sequence from both the candidates was homologous to the predicted sequence showing that the *aph* gene had inserted as predicted between base pairs 297 and 1326 (Appendix 1, Figures 3 and 4).

To confirm that transformants were *S. Typhimurium* two putative gene disrupted mutants were cultured on XLD agar for 48 hours at 37°C and produced colonies with black centres. Furthermore they both produced white/colourless colonies on MacConkey agar. Both candidates were Gram-negative rod shaped bacteria when Gram stained and observed

Figure 3.1 Verification of gene disruption by PCR



For each PCR (a-d):

Lane 1 = Candidate 1

Lane 2 = Candidate 2

Lane 3 = Positive control (a = L109 and b,c,d = SL1344)

Lane 4 = Negative control

	PCR number (see Table 2.5)	Description	Lane	Predicted Fragment Size (bp)	Actual Fragment Size (bp)
a	8	Internal <i>aph</i> check	1	471	~500
			2	471	~500
			3	471	~500
			4	0	0
b	2	External <i>acrA</i> check	1	2033	~2000
			2	2033	~2000
			3	1528	~1500
			4	0	0
c	6	<i>acrA aph</i> check	1	795	~800
			2	795	~800
			3	0	0
			4	0	0
d	7	<i>aph acrA</i> check	1	1148	~1100
			2	1148	~1100
			3	0	0
			4	0	0

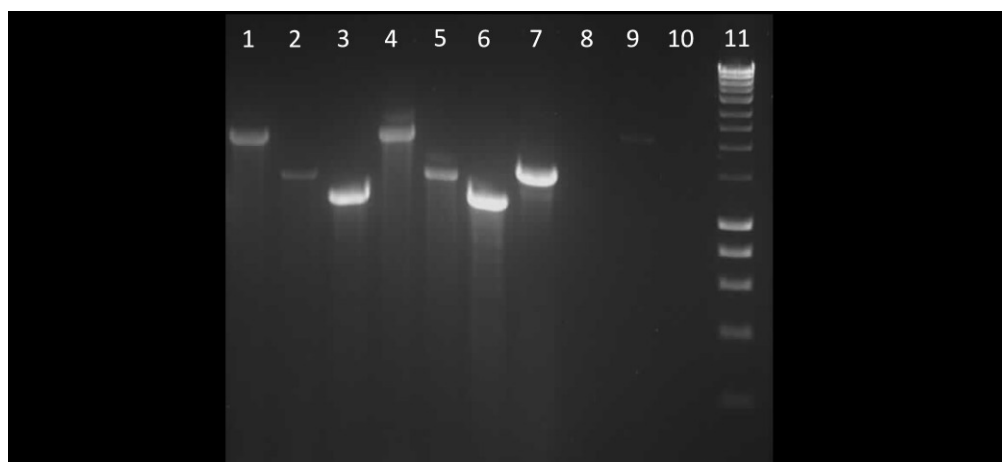
microscopically. Finally, Analytical profile indexing (API, Biomerieux, France) gave the identity of the bacteria to be *Salmonella spp.* with a probability of 98.9%. One *acrA::aph* mutant was retained for further study and given the laboratory code L823.

3.3.2 P22 transduction of the disrupted region

The λ red recombinase used during the construction of the gene disrupted mutants is known to induce non-specific mutations throughout the genome, albeit at a low frequency. To minimise this risk the disrupted region containing the *aph* cassette was transduced with P22 phage into the wild-type SL1344 background.

Following transduction with the P22 phage, two colonies were present on the kanamycin containing agar onto which the transduction reaction containing 50 μ l of P22 phage had been plated. Cell lysates were prepared from each putative transductant and in the first instance a PCR to assess the size of the *acrA* region was performed (Table 2.5, PCR 2). Both putative transformants produced an amplicons of approximately 2000 bp while the wild-type control, SL1344, produced an amplicon of approximately 1500 bp. Data for the transductants indicated that the disrupted region, containing the *aph* cassette, had been transduced from L823 into SL1344. Two further PCRs which combined external check primers with primers internal to the *aph* cassette produced amplicons of the predicted size confirming that the transduction had been successful (Table 2.5, PCRs 5 and 6) (Figure 3.2). One candidate was chosen and designated L884.

Figure 3.2 Confirmation of successful transduction of the disrupted *acrA::aph* region.



Lane	Strain	PCR number (see Table 2.5)	Predicted Fragment Size (bp)	Actual Fragment Size (bp)	Result	Conclusion
1	Candidate 1	1	2033	2033	<i>acrA</i> disrupted by <i>aph</i>	The <i>acrA::aph</i> region has been successfully transduced in L354
2		5	1355	1355	<i>aph</i> is present within <i>acrA</i>	
3		6	1148	1148	<i>aph</i> is present within <i>acrA</i>	
4	Candidate 2	1	2033	2033	<i>acrA</i> disrupted by <i>aph</i>	The <i>acrA::aph</i> region has been successfully transduced in L354
5		5	1355	1355	<i>aph</i> is present within <i>acrA</i>	
6		6	1148	1148	<i>aph</i> is present within <i>acrA</i>	
7	SL1344	1	1528	1528	Wild type <i>acrA</i>	The wild type control behaved as expected
8		5	0	0	<i>aph</i> is not present	
9		6	0	0	<i>aph</i> is not present	

Three PCR checks were performed on two candidate transductants and SL1344 was included as a control. Lane 10 contains a negative control and lane 11 contains a DNA sizing ladder which is labelled to the left hand side. The details of the PCR reactions are given in Table 2.5.

3.3.3 Removal of the *aph* cassette using the pCP20 plasmid

Removal of the *aph* gene from the gene disrupted mutant L884 (*acrA::aph*) was necessary to ensure that there were no polar effects from the insertion of this gene.

After L884 was transformed with pCP20, multiple large colonies appeared on all plates while the negative control, to which no plasmid was added, produced no colonies. After the plasmid was cured by overnight growth at 43°C all colonies grew well on antibiotic free LB agar. No growth was observed on LB agar containing ampicillin, confirming the plasmid had been lost. There was also no growth on LB agar containing kanamycin, indicating that the *aph* cassette had been successfully removed.

Three candidate transformants were selected. In a PCR to assess the size of the *acrA* region in each candidate (Table 2.5, PCR 2), candidates two and three produced amplicons of approximately 500 bps indicating the antibiotic resistance cassette had been removed while candidate one produced a fragment of 2033 bp indicating that the *aph* gene was still present (Figure 3.3 a and b). Finally, in a PCR using primers internal to the *aph* cassette (Table 2.5, PCR 7) an amplicon of approximately 500 bps was produced from L884 (*acrA::aph*) and candidate one, but no product was produced from candidates two or three confirming neither of them retain the *aph* cassette (Figure 3.3). Candidate two was selected and designated L976 (Δ *acrA*).

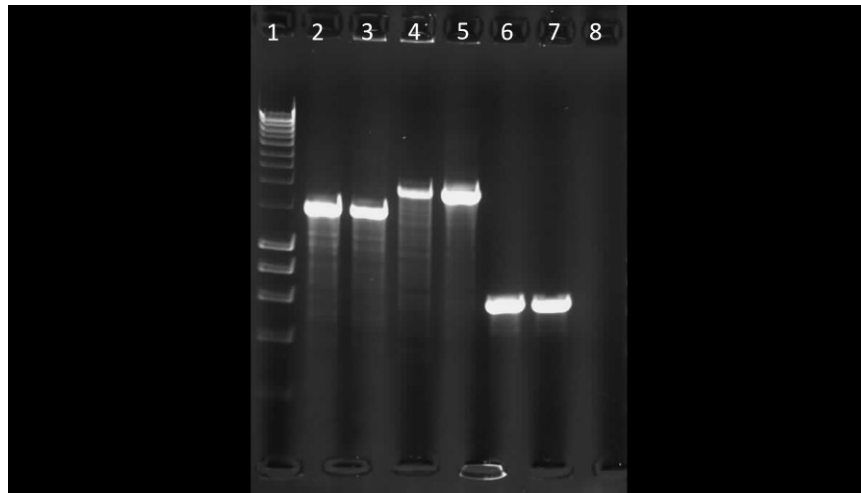
3.4 Complementation of *acrA* in the *acrA::aph* mutants

To ensure that the phenotypic changes in the *acrA* mutants (L823 and L884) were due to inactivation of *acrA*, the non-functional gene was complemented with the wild-type version

Figure 3.3 Confirmation of the removal of the *aph* cassette after treatment with the pCP20 plasmid.

a. PCR to assess the size of the *acrA* region (Table 2.5, PCR 2).

Lane 1 contains a DNA sizing ladder



Lane	Strain	Predicted Fragment Size (bp)	Actual Fragment Size (bp)	Conclusion
2	SL1344	1528	1528	Wild type <i>acrA</i>
3	L110 (<i>acrB::aph</i>)	1528	1528	Wild type <i>acrA</i>
4	L823 (<i>acrA::aph</i>)	2033	2033	<i>acrA</i> disrupted by <i>aph</i>
5	Candidate 1	539	2033	<i>acrA</i> disrupted by <i>aph</i>
6	Candidate 2	539	539	<i>aph</i> removed.
7	Candidate 3	539	539	<i>aph</i> removed.
8	Negative Control	0	0	Negative

b. PCR to confirm presence or absence of *aph* gene (Table 2.5, PCR 7).



Lane	Strain	Predicted Fragment Size (bp)	Actual Fragment Size (bp)	Conclusion
2	SL1344	0	0	Does not contain <i>aph</i>
3	L110 (<i>acrB::aph</i>)	471	471	Contains the <i>aph</i> gene
4	L823 (<i>acrA::aph</i>)	471	471	Contains the <i>aph</i> gene
5	Candidate 1	0	471	Contains the <i>aph</i> gene
6	Candidate 2	0	0	<i>aph</i> removed.
7	Candidate 3	0	0	<i>aph</i> removed.
8	Negative Control	0	0	Negative

in the gene disrupted mutants. This was done using the low copy number vector pWKS30 to try to achieve approximately wild-type levels of *acrA* expression (Wang and Kushner, 1991; Buckley *et al.*, 2006). After complementation, reversion to the wild-type phenotype would indicate that the inactivation of the target gene, *acrA*, and not polar effects, was responsible for the phenotypic change.

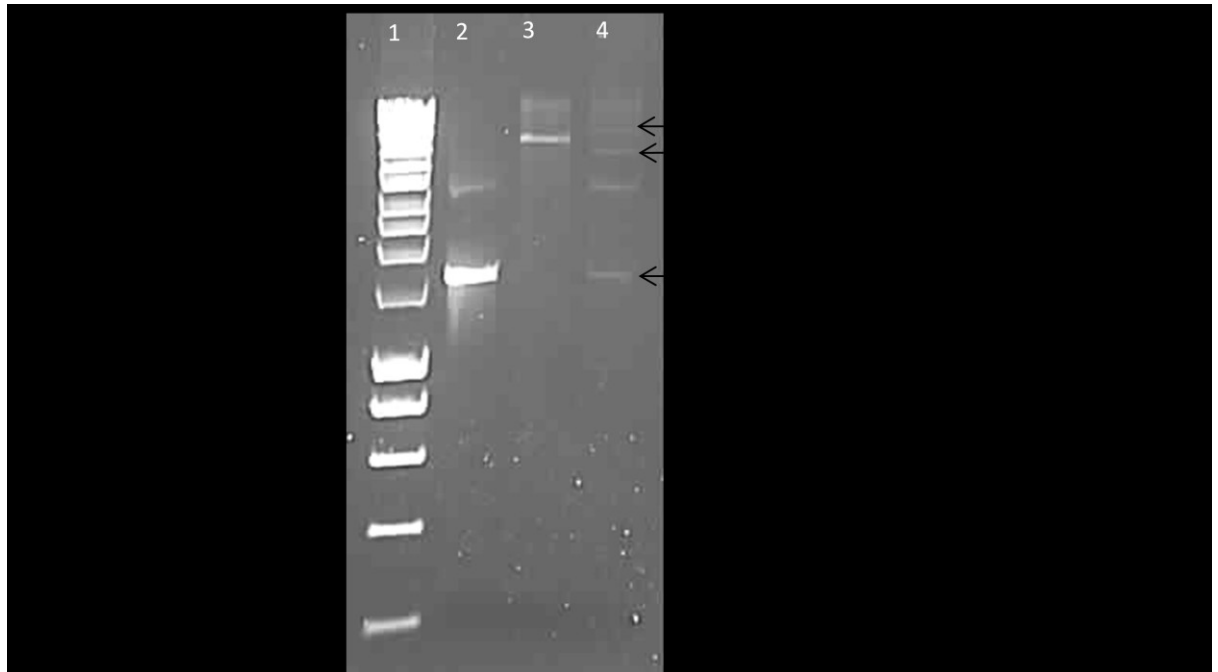
3.4.1 Construction of the complementation plasmid

Wild-type *acrA*, along with its native promoter, was amplified by PCR (Table 2.5, PCR 8) producing an amplicon of approximately 1500 bp. This product was digested with HindIII and BamHI (Figure 3.4, lane 2). The pWKS30 vector (Wang and Kushner, 1991) was successfully isolated from *E. coli* I906 and after digestion with HindIII and BamHI a linear product of 5.4 Kb was produced (Figure 3.4, lane 3). The plasmid and insert were successfully ligated producing a band of 6.9 Kb after agarose gel electrophoresis (Figure 3.4, lane 4). Bands of 5.4 Kb and 1542 bp were also present representing residual plasmid and insert which had not been incorporated into the ligated product (Figure 3.4, lane 4).

3.4.2 Transformation of strains with pWKS30*acrA*

After the ligated product was electroporated into *E. coli* DH5- α cells, three large colonies were produced on LB agar containing 50 μ g/ml ampicillin. Plasmid was recovered from all three putative transformants. After digestion with HindIII and BamHI only candidate two produced bands of 5.4 Kb and 1542 bp representing plasmid and insert respectively (Figure 3.5). A PCR for *acrA* (Table 2.5, PCR 2), using DNA isolated from the band representing the insert from candidate two as the template, produced an amplicon of approximately 1.5 Kb confirming the plasmid insert was *acrA* (Figure 3.6). The *E. coli* strain containing the

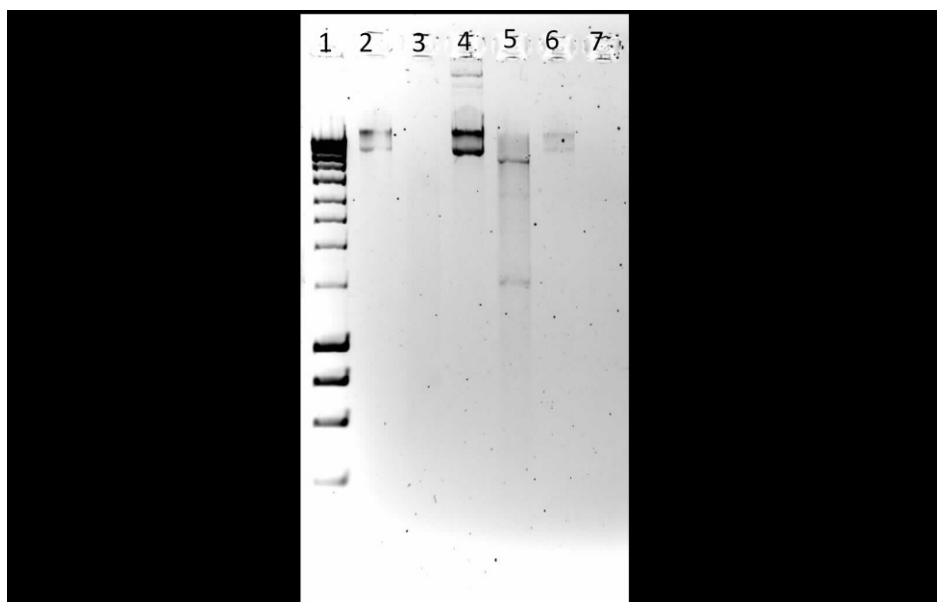
Figure 3.4 Construction of the *acrA* complementation plasmid by insertion of wild type *acrA* into the pWKS30 vector: ligation of digested insert and digested plasmid to give the ligated product, pWKS30*acrA*



Lane	Description	Expected fragment size (bp)	Actual fragment size (bp)
1	DNA sizing ladder (Bioline)	-	-
2	Digested Insert	1542	1542
3	Digested Plasmid	5400	5400
4	Ligation reaction	6900	6900

Lane one contains a DNA sizing ladder. In lane four, containing the ligation reaction, the ligated product is visible (6.9 Kb). Residual plasmid (5.4 Kb) and insert (1542 bp) are also visible which were not incorporated into the ligated product.

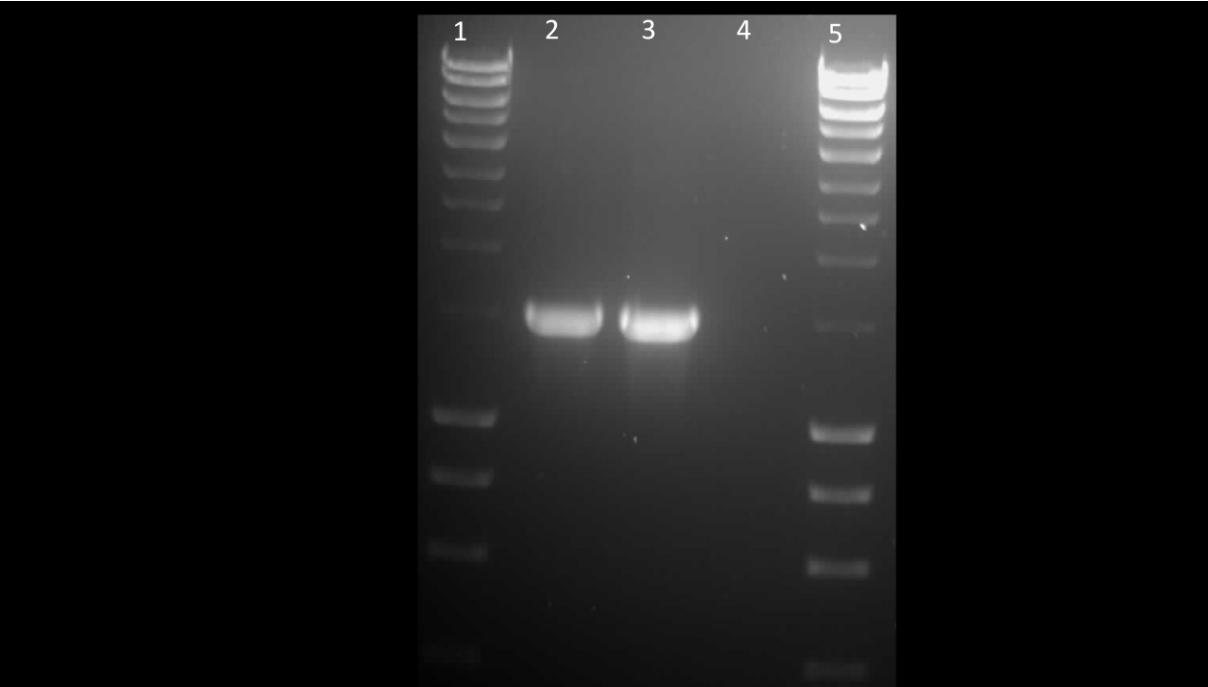
Figure 3.5 Confirmation of successful transformation of I750 with pWKS30 *acrA* plasmid: Undigested and digested plasmid recovered from three candidate transformants.



Lane	Description	Predicted Fragment Size (bp)	Actual Fragment Size (bp)	Conclusion
1	DNA sizing ladder	-	-	-
2	Candidate 1 Undigested plasmid	-	-	Plasmid isolated
3	Candidate 1 digested plasmid	5400 and 1542	No products	No bands after digestion
4	Candidate 2 Undigested plasmid	-	-	Plasmid isolated
5	Candidate 2 digested plasmid	5400 and 1542	5400 and 1542	Plasmid and insert visible after digestion
6	Candidate 3 Undigested plasmid	-	-	Plasmid isolated
7	Candidate 3 digested plasmid	5400 and 1542	No products	No band after digestion

Only the plasmid isolated from candidate two gave two bands of expected size after digestion. No expected fragment sizes are listed for the undigested plasmid as they cannot be accurately sized until linearised. The gel is shown as black bands on a white background as the bands were difficult to see.

Figure 3.6 Confirmation by PCR that the insert in pWKS30 is *acrA*



Lane	Description	Predicted Fragment Sizes (bp)	Actual Fragment Sizes (bp)	Conclusion
1, 5	DNA sizing ladder	-	-	-
2	Candidate 2	1528	1528	<i>acrA</i> amplified from purified plasmid insert
3	SL1344	1528	1528	<i>acrA</i> amplified
4	Negative control	0	0	Negative

After isolation and digestion of the plasmids (Figure 3.5) the band representing the plasmid insert was excised and the DNA isolated. This DNA was used as the template in this PCR reaction. The band of around 1500bp from candidate two produced the same size product as the positive control, SL1344, so contains the *acrA* insert.

pWKS30*acrA* plasmid was designated I750. The pWKS30*acrA* plasmid was successfully recovered from I750 and electroporated into L823 (*acrA::aph*), L884 (transduced *acrA::aph*), and SL1344. Transformation was confirmed by isolation and digestion of the plasmid with HindIII and BamHI followed by a PCR for *acrA* to confirm insert identity. These strains were designated L975, L979, and L980, respectively.

3.5 Expression of *acrB*/AcrB in mutants lacking *acrA*

The *acrA* and *acrB* genes exist together in a single operon. To determine the role of AcrA alone it is crucial that expression of *acrB*/AcrB was un-affected by the disruption of the *acrA* region. Reverse transcriptase PCR was used to confirm *acrB* transcription and Western Blotting was used to confirm the presence of the AcrB protein.

3.5.1 Reverse transcriptase (RT) PCR to measure *acrA* and *acrB* transcription

After RNA isolation all samples were electrophoresed on a 1% agarose gel confirming RNA had been successfully isolated from all three biological replicates of SL1344, L884 (*acrA::aph*), L976 (Δ *acrA*), L979 (L884 with pWKS30*acrA*) and L110 (*acrB::aph*), respectively. A PCR for 16s was performed to determine whether any was DNA present (Table 2.5, PCR 5). No product was produced from any of the samples indicating that no DNA was present.

After cDNA was synthesised from the isolated RNA, PCRs using primers internal to the genes of interest, *acrA* and *acrB*, were set up along with a control PCR using primers internal to 16s (Table 2.5, PCRs 3, 4, and 5). The levels of the PCR products were quantified according to their peak areas after denaturing high-pressure liquid chromatography (dHPLC) on the WAVE DNA fragment analysis system and subsequent analysis with the NAVIGATOR (version 1.4)

software (Transgenomic Inc.) as described by Eaves *et al.*, (2004). Expression levels of each gene were normalised against levels of *16s* in each sample.

The *acrA* transcript was detected in SL1344 but was absent in L884 (*acrA::aph*) (Figure 3.7 a). A small amount of transcript was detected in L976 (Δ *acrA*) but this was significantly lower than in SL1344 and was not significantly different from L884 (*acrA::aph*). The *acrA* transcript was slightly over-expressed in the complemented strain L979 (*acrA::aph*, pWKS30*acrA*) (Figure 3.7 a).

The level of *acrB* detected in the *acrA* mutants (L884 and L976) was not significantly different to the level detected in SL1344 (Figure 3.7 b). No *acrB* expression could be detected in L110 (*acrB::aph*) (Figure 3.7 b).

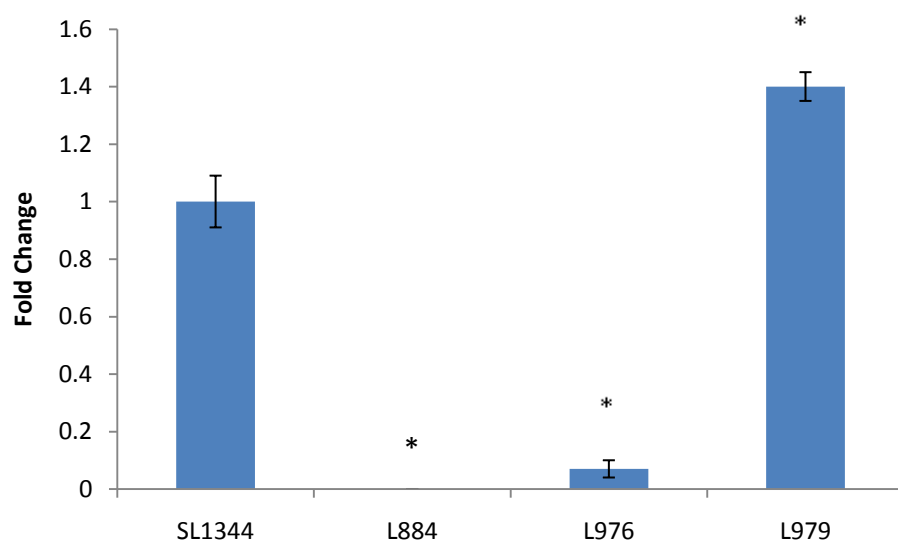
3.5.2 Western Blotting to detect the presence of AcrA, AcrB and TolC proteins

Western blotting with anti-AcrA antibody confirmed the expression of the AcrA protein in SL1344 and L110 (*acrB::aph*) with a band present at ~42 kDa. Expression was absent in both AcrA mutants (L884 (*acrA::aph*) and L976 (Δ *acrA*)) and was over-expressed by 3.2 fold in the complemented strain (L979). Surprisingly, the AcrA protein could not be detected in the *tolC* mutant (L109) (Figure 3.8a).

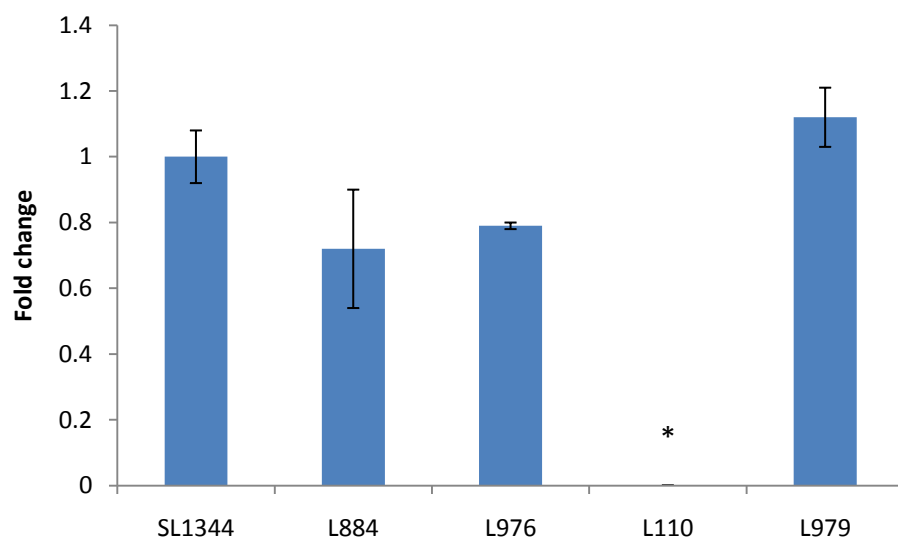
Western blotting with anti-AcrB antibody confirmed the expression of the AcrB protein in SL1344, L884 (*acrA::aph*) and L109 (*tolC::aph*) with a band present at ~110-120 kDa representing the AcrB monomer. Expression was absent in the *acrB* mutant (L110) (Figure 3.8b).

Figure 3.7 RT-PCR to measure *acrA* and *acrB* expression

- a. Expression of *acrA* in SL1344, L884 (*acrA::aph*), L976 (Δ *acrA*) and L979 (*acrA::aph*, pWKS30*acrA*)

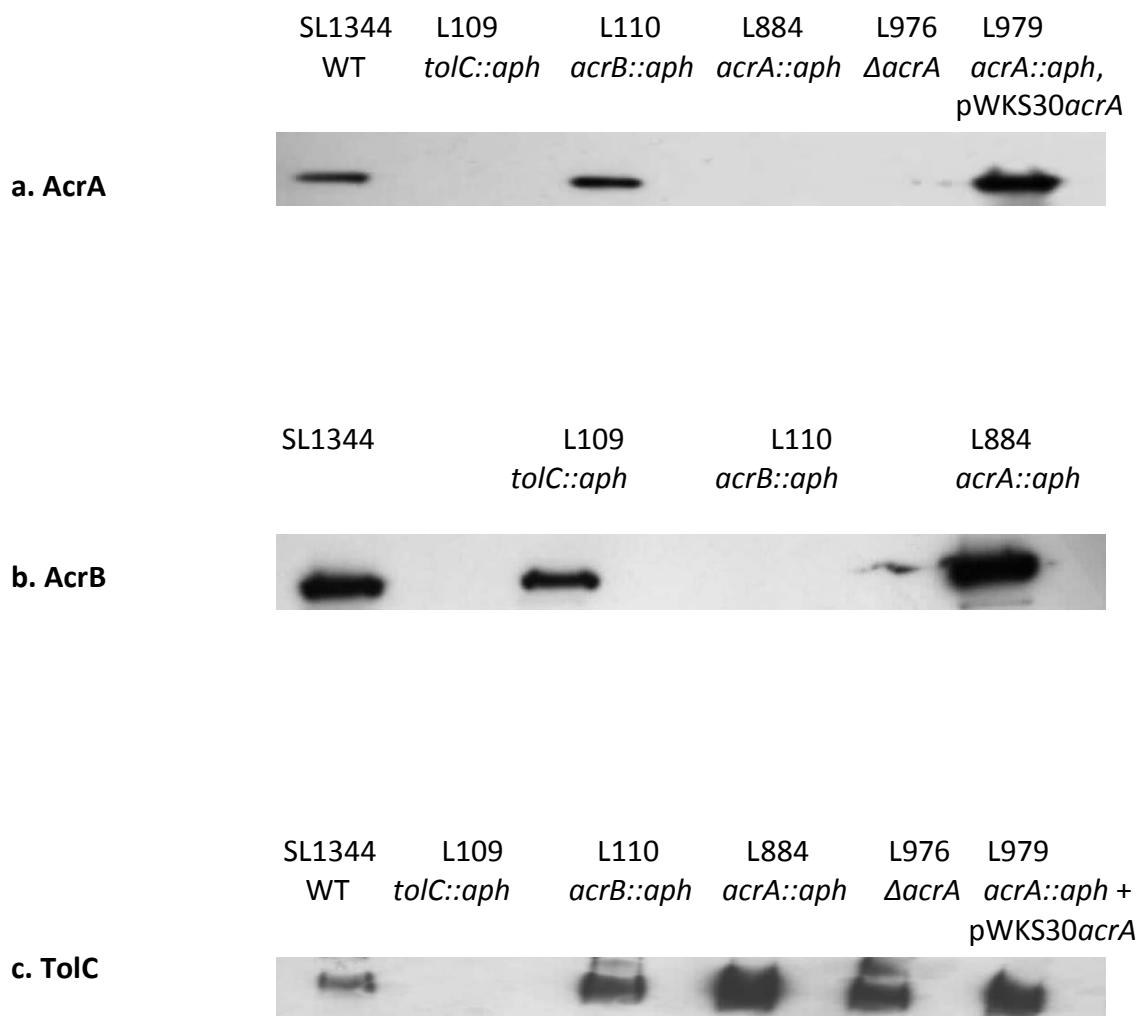


- b. Expression of *acrB* in SL1344, L884 (*acrA::aph*), L976 (Δ *acrA*), L110 (*acrB::aph*) and L979 (*acrA::aph*, pWKS30*acrA*)



Data shown are presented as fold change compared to SL1344 +/- standard deviation and are the mean of three independent experiments. Results that were significantly different from SL1344 are denoted by *.

Figure 3.8 Western Blotting for AcrA, AcrB and TolC in SL1344 and efflux mutants thereof.



Membrane protein preparations were blotted with anti-AcrA, anti-AcrB or anti-TolC antibodies.

Western blotting with anti-TolC antibody confirmed the expression of the TolC protein in SL1344, the *acrB* mutant (L110), the *acrA* mutants (L884, L976) and the *acrA* complemented strain (L979) with a band present at ~150 kDa representing the TolC trimer. Expression was absent in the *tolC* mutant (L109) (Figure 3.8c).

3.6 Phenotypic Characterisation of *S. Typhimurium* mutants lacking AcrA

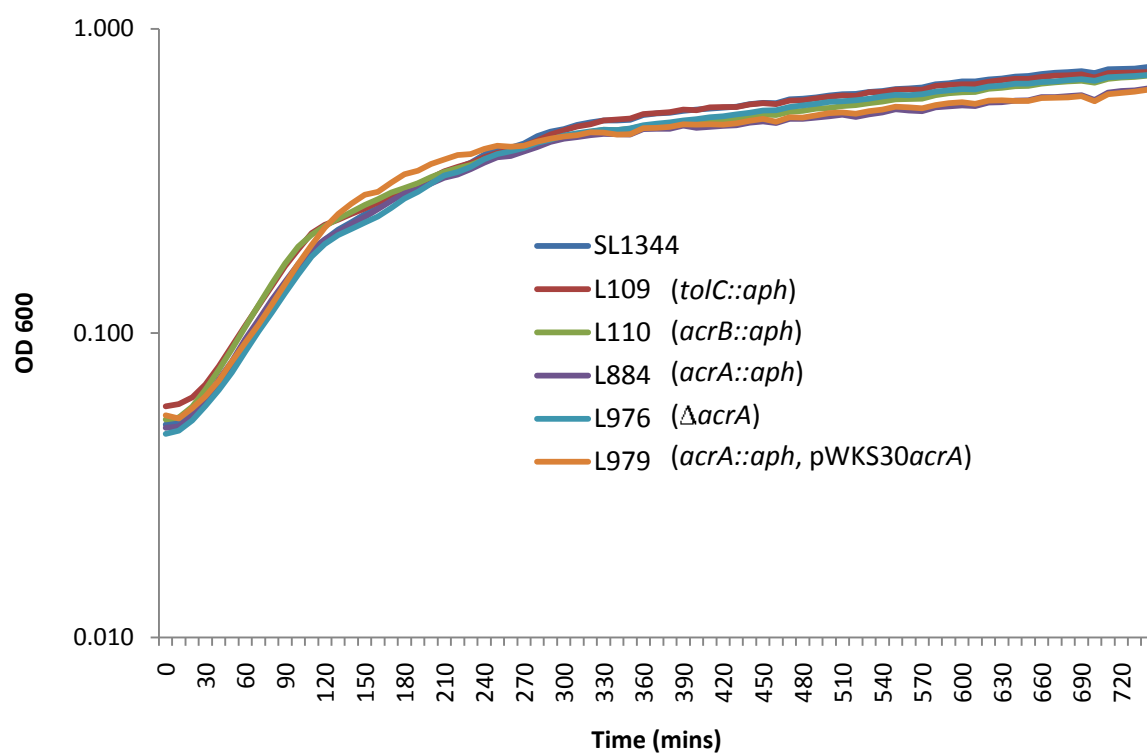
3.6.1 Bacterial growth kinetics in LB broth

There was no significant difference between the generation times or the final optical density at stationary phase for SL1344, L109 (*tolC::aph*), L110 (*acrB::aph*), L884 (*acrA::aph*), L976 (Δ *acrA*) or L979 (*acrA::aph* with pWKS30*acrA*) in LB broth (Figure 3.9). However, it has also been shown by this laboratory that mutants lacking *acrA* or *acrB* are impaired in their ability to grow in anaerobic conditions after both 8 and 24 hours compared to SL1344. The ability of the mutant lacking *tolC* to grow anaerobically was not affected (Webber *et al.*, 2009).

3.6.2 Hoechst 33342 (Bis-benzamide) accumulation

Hoechst H33342 is a dye which fluoresces when bound to DNA and therefore its accumulation can be used to assess relative levels of active efflux (Webber *et al.*, 2008; Webber and Coldham, In Press). Accumulation of Hoechst H33342 in the *acrA* mutant was determined and compared to that of SL1344. Inactivation of *acrA* conferred a two-fold increase in accumulation compared to the wild-type strain SL1344 ($P = 0.04$). This phenotype was abolished in the complemented strain (L979). The *acrB* mutant accumulated 2.9 fold more Hoechst H33342 ($P = 0.005$), while the *tolC* mutant accumulated 3.6 fold more ($P = 0.003$) (Figure 3.10).

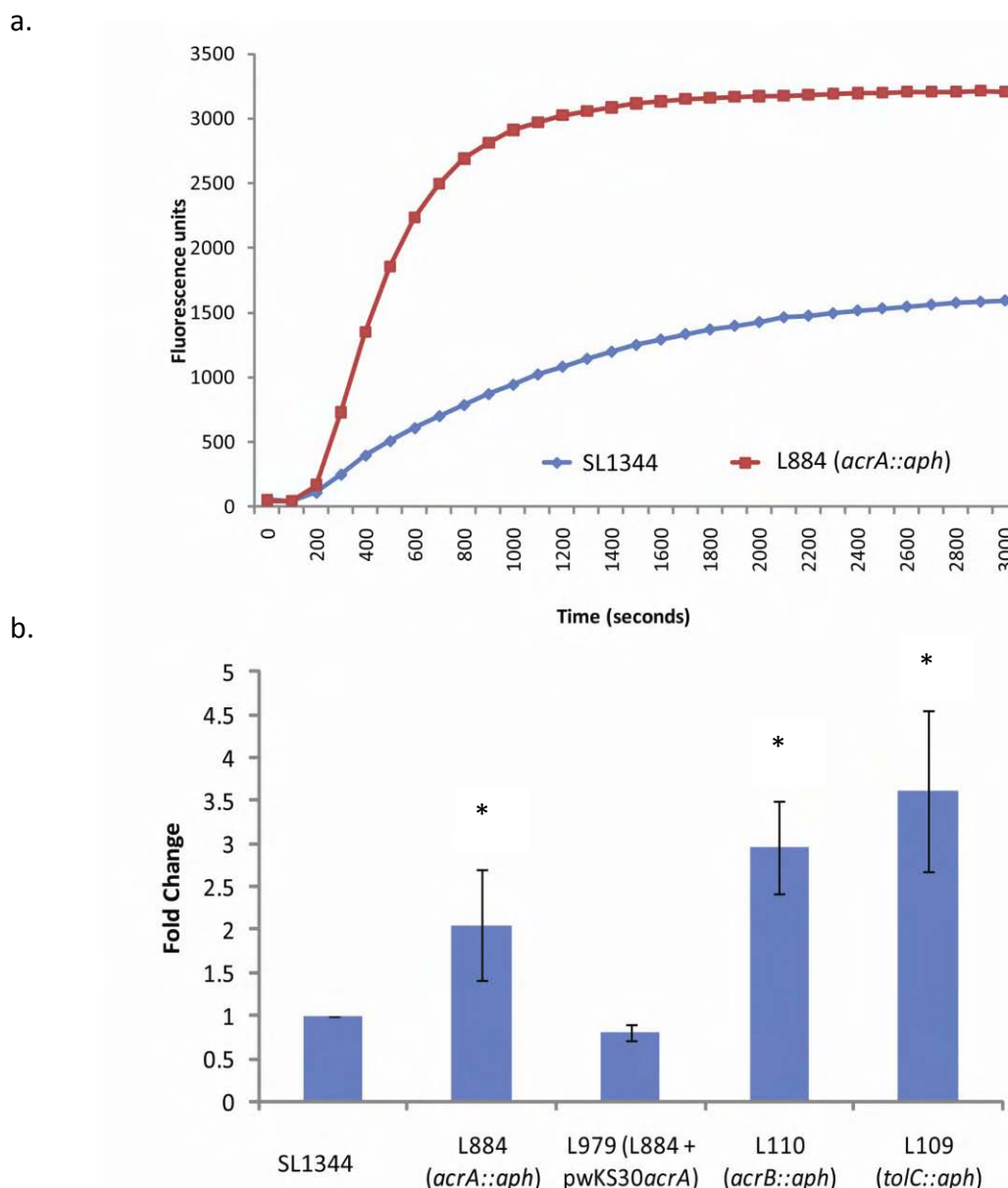
Figure 3.9 (a) Growth kinetics of SL1344 and efflux mutants thereof in LB broth at 37 °C for 12 hours



(b) Generation Times and Optical Density at Stationary Phase (\pm standard deviation)

Strain	Mean Generation Time (minutes)	T-test (<i>P</i>)	OD 600 at Stationary Phase	T-test (<i>P</i>)
SL1344	47.38 \pm 0.65	-	0.749 \pm 0.585	-
L109 (<i>tolC::aph</i>)	46.15 \pm 0.71	0.09	0.721 \pm 0.557	0.31
L110 (<i>acrB::aph</i>)	44.41 \pm 1.34	0.06	0.702 \pm 0.538	0.17
L884 (<i>acrA::aph</i>)	46.53 \pm 3.36	0.69	0.637 \pm 0.473	0.06
L976 (Δ <i>acrA</i>)	47.46 \pm 2.55	0.96	0.704 \pm 0.540	0.14
L979 (<i>acrA::aph</i> , pWKS30 <i>acrA</i>)	48.08 \pm 2.06	0.60	0.630 \pm 0.466	0.06

Figure 3.10 Accumulation of Hoechst H33342 (Bis-benzamide) by SL1344, L884 (*acrA::aph*), L979 (*acrA::aph*, *pWKS30acrA*), L110 (*acrB::aph*) and L109 (*tolC::aph*)



a. The accumulation of Hoechst H33342 in L884 (*acrA::aph*) and SL1344 over time

b. The fold change in accumulation of Hoechst H33342 in SL1344, the *acrA* mutant (L884), the *acrA* complemented strain (L979), the *acrB* mutant (L110) and the *tolC* mutant (L109).

The data presented is the mean of three separate experiments, each performed in duplicate. Data in b is presented as fold change compared to SL1344 at the end point of the time course shown in a +/- standard deviation. Student's t-tests were performed to compare the accumulation of each strain to that of SL1344 and those returning *P* values of less than 0.05 are indicated by *

3.6.3 Antimicrobial Susceptibility

S. Typhimurium SL1344 was susceptible to all 13 agents tested (Table 3.1). These agents were chosen as they are known substrates of AcrAB-TolC and represent different antibiotic classes as well as the dyes and detergents.

3.6.3.1 Antimicrobial Susceptibility of the *acrA* mutant, the transduced *acrA* mutant strain (L884) and the *acrA* mutant with *aph* removed (L976) (Table 3.1).

L823 (*acrA::aph*) and L884 (transduced *acrA::aph*) showed the expected increase in kanamycin resistance to >256 µg/ml due to incorporation of the *aph* gene. L976 (Δ *acrA*) was susceptible to kanamycin due to the removal of the *aph* gene. The MICs for the *acrA* mutant (L823), the transduced *acrA* mutant (L884) and the *acrA* mutant from which the *aph* gene has been removed (L976) were all within one dilution of each other.

MIC values for L109 and L110 were within one dilution of previously published data from this laboratory (Eaves *et al.*, 2004; Buckley *et al.*, 2006). Inactivation of *acrA* resulted in increased susceptibility to almost all agents tested except tetracycline, bile, SDS and Triton X-100. Inactivation of either *acrA*, *acrB* or *tolC* gave a similar increase (+/- one dilution) in susceptibility to ampicillin, nalidixic acid and chloramphenicol. However, the hypersusceptibility profiles produced by strains lacking *acrA* were distinct from those for the *acrB* or *tolC* mutants. Strains lacking *acrA* (L823, L884 and L976) were also as susceptible (+/- one dilution) as the *acrB* mutant (L110) to ethidium bromide and Triton X-100, but were more susceptible than L110 (*acrB::aph*) to ciprofloxacin, triclosan, acriflavine and fusidic acid. The *acrA* mutants (L823, L884 and L976) were also as susceptible (+/- one dilution) as L109 (*tolC::aph*) to acriflavine and fusidic acid while the *tolC* mutant was more susceptible to

Table 3.1 MICs of a range of antibiotics, dyes and detergents for the *acrA*, *acrB* and *tolC* mutants (L823, L110 and L109), the transduced *acrA* mutant strain (L884) and the *acrA* mutant with *aph* gene removed (L976) compared to SL1344.

		MIC · (µg/ml)												
	Genotype	Kan	Amp	Nal	Cip	Chl	Tet	Tric	EthBr	Acr	Fus	Bile	SDS	Triton X-100
SL1344		8	1	4	0.015	4	1	0.06	>256	256	>512	2048	>512	>2000 0
L823	<i>acrA::aph</i>	>256	0.25	0.5	0.002	0.5	0.5	0.008	64	16	4	2048	256	>2000 0
L884	Transduced L823	>256	0.25	0.5	0.002	0.5	0.5	0.008	64	16	4	2048	256	>2000 0
L976	Δ <i>acrA</i>	8	0.25	0.5	0.002	0.5	0.5	0.008	64	16	4	2048	256	>2000 0
L109	<i>tolC::aph</i>	256	0.25	1	0.015	1	0.5	0.015	16	16	4	256	64	1200
L110	<i>acrB::aph</i>	256	0.25	1	0.015	1	1	0.03	64	64	16	1024	128	>2000 0

Red text indicates a ≥ 2 fold decrease in MIC value compared with SL1344.

Kan, kanamycin; amp, ampicillin; Nal, nalidixic acid; Cip, ciprofloxacin; Chl, chloramphenicol; Tet, tetracycline; Tric, triclosan; EtBr, ethidium bromide; Acr, acriflavine; Fus, fusidic acid, SDS, sodium dodecylsulphate; Triton, triton X-100.

ethidium bromide, bile, SDS and Triton X-100. The *acrA* mutant was more susceptible than L109 (*tolC::aph*) to ciprofloxacin and triclosan.

3.6.3.2 Antimicrobial Susceptibility of the strains containing the pWKS30 vector alone (L917, L918 and L996) and strains containing pWKS30 *acrA* (L980, L975 and L979) (Table 3.2).

SL1344, L823 (*acrA::aph*) and L884 (*acrA::aph*) were transformed with the pWKS30 vector giving strains L917, L918 and L996, respectively and with pWKS30*acrA* giving strains L980, L975 and L979, respectively. Strains containing either the pWKS30 plasmid (L917, L918 and L996) or the pWKS30 *acrA* plasmid (L980, L975 and L979) were ampicillin resistant (MIC = >128 µg/ml) due to the presence of the ampicillin resistance gene on the pWKS30 plasmid. Apart from ampicillin, the MICs of antibiotics, dyes and detergents for L917 (SL1344 with pWKS30), L918 (L823 with pWKS30 vector) and L996 (L884 with pWKS30 vector) were all within one dilution of those for the parent strain without the plasmid showing that possession of the pWKS30 plasmid had no inherent effects on antimicrobial susceptibility. The MICs of antibiotics, dyes and detergents for L980 (SL1344 with pWKS30*acrA*) were all within one dilution of those for SL1344 without the plasmid. Except for kanamycin and ampicillin, the MICs for the *acrA* mutants carrying the complementation plasmid (L975 and L979) were all within one dilution of those for the wild-type SL1344 showing that complementation of *acrA* has restored the antimicrobial susceptibility profile of the mutants to wild-type levels.

Table 3.2 MICs of a range of antibiotics, dyes and detergents for the *acrA* mutant and transduced mutant (L823 and L884), strains containing the pWKS30 vector alone (L917 and L918) and strains containing pWKS30 *acrA* (L980, L975 and L979)

		MIC · (µg/ml)											
	Genotype	Kan	Amp	Nal	Cip	Chl	Tet	Tric	EthBr	Acr	Fus	Bile	SDS
SL1344	WT	8	1	4	0.015	4	2	0.06	>256	256	>512	>1024	>512
L917	SL1344 with pWKS30 vector	8	>128	4	0.015	2	2	0.06	>256	256	512	>1024	>512
L980	SL1344 with pWKS30 <i>acrA</i>	8	>128	4	0.015	4	2	0.06	>256	256	512	>1024	>512
L823	<i>acrA::aph</i>	>256	0.25	0.5	0.008	1	0.5	0.008	64	16	4	2048	512
L918	L823 with pWKS30 vector	>256	>128	1	0.008	0.5	0.5	0.015	64	16	4	>1024	256
L975	L823 with pWKS30 <i>acrA</i>	>256	>128	4	0.03	4	2	0.06	>256	256	512	>1024	>512
L884	Transduced L823	>256	0.25	0.5	0.008	0.5	0.5	0.008	64	16	4	>1024	256
L996	L884 with pWKS30 vector	>256	>128	0.5	0.008	0.5	0.5	0.008	64	16	4	>1024	256
L979	L884 with pWKS30 <i>acrA</i>	>256	>128	4	0.03	4	2	0.06	>256	256	512	>1024	>512

Red text indicates a ≥ 2 fold decrease in MIC value compared with SL1344. Kan, kanamycin; Nal, nalidixic acid; Cip, ciprofloxacin; Chl, chloramphenicol; Tet, tetracycline; Tric, triclosan; EtBr, ethidium bromide; Acr, acriflavine; Fus, fusidic acid, SDS, sodium dodecylsulphate; Triton, triton X-100.

3.6.4 Transmission electron microscopy

SL1344 and the *acrA*, *acrB* and *tolC* mutants were grown overnight in LB broth before being washed, fixed with 2.5% glutaraldehyde and viewed by transmission electron microscopy by Bill Cooley at the Veterinary Laboratories Agency, Weybridge, Surrey. The size and appearance of the *acrA* mutant (L884) was not significantly different to the wild-type SL1344 (Figure 3.11 a and b).

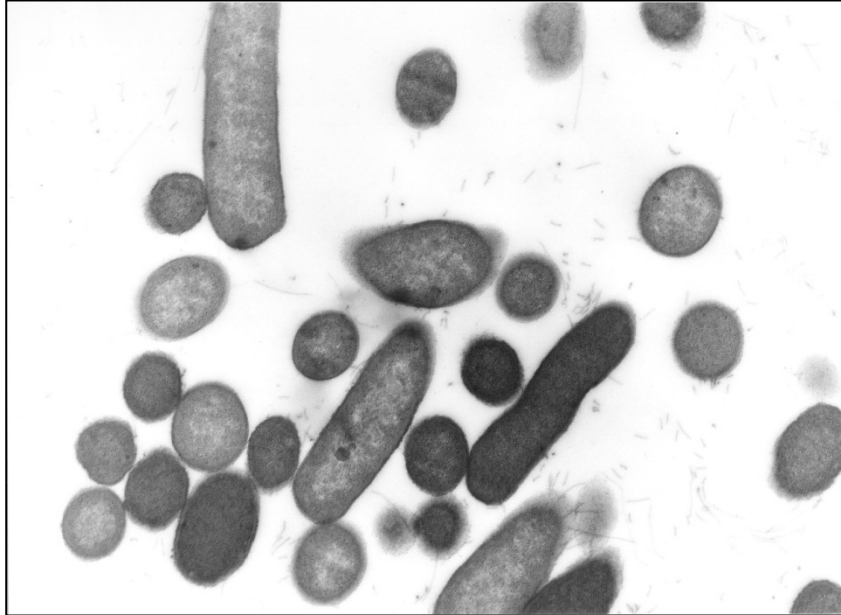
3.7 Discussion

The *acrA* gene of *Salmonella* Typhimurium, was successfully inactivated by insertion of the *aph* gene (L823) and this mutation was successfully transduced (L884), the *aph* gene removed (L976) and the mutation complemented (L979). RT-PCR confirmed that *acrA* was not transcribed in any of the *acrA* mutants and was transcribed at slightly higher than wild-type levels in the complemented strain (L979). Western blotting showed the same pattern of expression for the AcrA protein. Importantly, RT-PCR and Western blotting showed that *acrB* and AcrB, respectively were expressed in the *acrA* mutants at the same level as in the wild-type strain and TolC was present in all strains except the *tolC* mutant. This is crucial as it shows, that although *acrA* and *acrB* are in the same operon, that inactivation of *acrA* has not adversely affected *acrB* expression so the loss of the periplasmic adaptor component alone can be studied.

An interesting result from the Western Blotting was that AcrA was not detected in the TolC mutant although previous work in this laboratory has shown that *acrA* transcription was not affected by inactivation of *tolC* (Webber *et al.*, 2009). The complete structure of the assembled *E. coli* AcrAB-TolC system was recently published and this shows the structural

Figure 3.11 Transmission Electron Microscopy to examine bacterial structure

a. SL1344



b. L884 (*acrA::aph*)



Images taken by Bill Cooley at the Veterinary Laboratories Agency, Weybridge, Surrey

importance of the AcrA protein in stabilising the complex between the weakly interacting inner and outer membrane components, AcrB and TolC (Symmons *et al.*, 2009). A further study has confirmed the importance of *E. coli* AcrA, in particular the C-terminal domain, in assembly of the efflux pump (Ge *et al.*, 2009). Symmons and colleagues suggest that AcrA may bind to AcrB and that this complex then recruits the outer membrane component, TolC. One hypothesis for the unusual observation in the TolC mutant in this study is that if the AcrAB complex is unable to recruit the outer membrane component (because it is absent) then the periplasmic component may be degraded by periplasmic proteases. The study by Ge *et al.*, (2009) provides some evidence for this as the C-terminus of AcrA is readily cleaved by trypsin when either AcrB or TolC is absent, or when AcrA is linked to either AcrB or TolC alone in a bi-partite complex, but is protected from proteolysis by assembly of the tri-partite system (Ge *et al.*, 2009). It is therefore possible that, *in vivo*, assembly of the tri-partite system protects AcrA from degradation by periplasmic proteases but when TolC is not present, meaning that the complex cannot form, that AcrA is degraded. In wild-type cells degradation of un-complexed AcrA is probably important in preventing periplasmic overcrowding as over-expression of AcrA in mutants lacking AcrEF is toxic, and results in cell division defects (Lau and Zgurskaya, 2005). Although this is not a specific effect of AcrA *per se* but an effect of periplasmic overcrowding in this mutant, it suggests that prevention of periplasmic over-crowding by proteolysis of unused proteins is probably important in maintaining normal cellular function. Further study is required to explore this hypothesis and to explain the lack of AcrA detected in the TolC mutant and this is discussed in section 3.8.

Inactivation of *acrA*, *acrB* or *tolC* did not affect the growth of *S. Typhimurium* in LB broth in aerobic conditions but a separate study from this laboratory showed that growth of the *acrA*

and *acrB* mutants was impaired in anaerobic conditions (Webber *et al.*, 2009). In the *acrB* mutant this impaired anaerobic growth has been linked to reduced transcription of the *nir*, *nar* and *nap* operons which encode genes known to be involved in anaerobic respiration. In contrast, these genes were shown to be transcribed at higher levels in the *acrA* mutant than in SL1344 so the reason for the defect in anaerobic growth in this strain is not known (Webber *et al.*, 2009).

The method used to study the growth of the efflux mutants compared to SL1344 (described in 2.7.1) is an automated system allowing high throughput and generation of multiple biological and technical replicate data sets. However, this method has limitations. For example, optical density was used to measure growth rather than the more accurate method of using viable counts. This is a notable limitation of the study. However, previous work in this laboratory has confirmed the same findings for these strains by viable counting and by estimation of total protein content (Mark Webber, unpublished work; Webber *et al.*, 2009 supplementary material). A further limitation of the method is that no initial lag phase was seen before entry into the logarithmic phase of growth due to the high initial inoculum. Therefore, while there was no significant difference in generation time or final optical density, it is not possible to comment whether the efflux mutants have a lag phase which is significantly different from that of the wild-type strain, SL1344. In addition, the growth experiments described in section 3.6.1 were performed in LB broth while later experiments used the tissue culture media MEM and DMEM so the results discussed here cannot necessarily be extrapolated to be relevant during later experiments.

Inactivation of *acrA* in *Salmonella* Typhimurium SL1344 led to increased susceptibility to a range of antibiotics, dyes and detergents compared with the parental strain *S. Typhimurium* SL1344. This hypersusceptibility phenotype was abolished by complementation with pWKS30*acrA*. When the susceptibility of the *acrA* mutant was compared with data for SL1344 in which *acrB* or *tolC* had been inactivated subtle gene dependant differences in phenotype were seen. The susceptibility of the *acrA* mutant (L884) to the antimicrobials tested was similar to the *acrB* mutant for 5 of the 13 agents tested although the *acrA* mutant was more susceptible than L110 to ciprofloxacin, triclosan, acriflavine and fusidic acid. The greatest overall increase in susceptibility was seen with the inactivation of *tolC*, particularly to fusidic acid, nalidixic acid and Triton X-100, as previously reported for both *Salmonella* and *E. coli* (Nishino *et al.*, 2003; Buckley *et al.*, 2006; Nishino *et al.*, 2006). Interestingly, the *acrA* mutant was more susceptible to ciprofloxacin and triclosan than the *tolC* mutant. This shows that AcrA plays an important role in efficient efflux of these antimicrobials.

The greater susceptibility of the *acrA* mutants to some agents may be partially explained by the reported promiscuity of the AcrA protein with other efflux proteins of the RND family of efflux pumps including AcrD and AcrF (Kobayashi *et al.*, 2001a; Elkins and Nikaido, 2002, 2003). It may also associate with efflux pumps of other families which can also transport ciprofloxacin and triclosan. The TolC protein is also promiscuous and acts as the outer membrane component for many efflux systems including other RND pumps (Nagakubo *et al.*, 2002; Hirakawa *et al.*, 2003; Nishino *et al.*, 2003; Nishino *et al.*, 2006) as well as members of the MFS (Nishino and Yamaguchi, 2002) and ABC transporter families (Kobayashi *et al.*, 2001b) therefore, disruption of TolC will not only affect the ability of the AcrAB pump to

function but will interfere with other export pathways possibly explaining its generally greater susceptibility.

Another possible explanation for the magnitude of susceptibility in the AcrA mutant is that AcrA may have some role in recruiting substrates into the pump from the periplasm (Zgurskaya *et al.*, 2008; Symmons *et al.*, 2009). The recently published structure of the assembled pump shows that, while AcrA does not make any contact with the binding cleft between PC1 and PC2 subdomains of AcrB, the newly modeled membrane proximal domain of AcrA binds very close to the binding pocket identified between the PN2 and PC1 subdomains of AcrB. The suggestion is that AcrA could therefore have a role in facilitating recruitment of hydrophobic substrates into this binding pocket (Symmons *et al.*, 2009). It has not been confirmed that the mutants produce typical LPS and therefore, the possibility that pleiotropic effects of an atypical LPS are contributing to the susceptibility phenotype cannot be ruled out.

Interestingly, a comprehensive study of multiple efflux pump mutants by Nishino and colleagues (2006) showed that a mutant lacking both AcrA and AcrB was hypersusceptible to antibiotics, dyes and detergents but to the same level as a strain lacking only AcrB. This is interesting as the results from this study suggest that lack of AcrA has a distinct susceptibility phenotype to loss of AcrB. However, this discrepancy is probably explained by the different agents tested in the two studies and, as the susceptibility is substrate specific, the difference between the single *acrB* mutant and the *acrAB* mutant may be detected if more agents were tested.

Accumulation of the fluorescent dye Hoechst 33342 was increased two fold in the *acrA* mutant (L884 *acrA::aph*) and to a greater extent in the *acrB* and *tolC* mutants showing a significant decrease in efflux. These data suggest that the PAP is required for efficient efflux. The observed decrease in efflux goes some way to explain the increase in susceptibility of strains lacking components of AcrAB-TolC to the antimicrobials tested. The decreased efflux means that toxic compounds are not extruded as efficiently and therefore accumulate inside the cell and are lethal at lower total concentration. However, the effect is substrate specific as the *acrA* mutant was the most susceptible to triclosan while it did not have the most attenuated efflux level.

Loss of functional *acrA* significantly affected the efflux activity and antibiotic susceptibility of SL1344 and importantly the mutant phenotype was distinct from strains lacking either AcrB or TolC. This suggests a key role for AcrA in efficient operation of this pump which is separate and distinct from that of AcrB (or other protein partners) or TolC. This is complementary to the growing body of structural data showing that AcrA physically interacts with both AcrB and TolC (Husain *et al.*, 2004; Tikhonova and Zgurskaya, 2004; Bavro *et al.*, 2008) and is structurally important in assembly of the complex (Symmons *et al.*, 2009). The extent of the change in phenotype is probably augmented by the promiscuity of AcrA such that disruption of *acrA* will also cause the function of other RND pumps to be impaired (Kobayashi *et al.*, 2001a; Elkins and Nikaido, 2002, 2003; Hirakawa *et al.*, 2003; Nishino *et al.*, 2003).

3.8 Further work

Limitations of the method used to measure growth of SL1344 and the efflux mutants have been discussed. To overcome these limitations the findings the experiments should first be confirmed by viable counts. A more dilute bacterial suspension or a range of dilutions could be used to study the effect of inactivation of these efflux proteins on the lag phase of growth and finally the experiments should be repeated in all media types relevant to the study including both MEM and DMEM.

Strains which over-express each of the components of AcrAB-TolC will be constructed along with a strain which over-expresses all three components of the efflux system. The hypothesis is that over-expression of single components or the entire efflux system may confer multi-drug resistance as over-expression of efflux pumps has been found to confer MDR in clinical isolates (Piddock *et al.*, 2000; Baucheron *et al.*, 2004).

Previous work by Nishino *et al.*, (2006) has included characterisation of a *Salmonella* Typhimurium strain lacking both AcrA and AcrB. Further work could include constructing mutants with inactivations in various combinations of RND pump genes including a strain that lacks all three components of AcrAB-TolC. In addition a strain which lacks AcrA and the other major RND periplasmic adaptor protein, AcrE could be constructed. It is postulated that the efflux activity of strains lacking multiple components of the RND efflux systems will be even more impaired and this would allow investigation of the level of redundancy in these systems.

The RND efflux pump AcrD is involved in the transport of aminoglycoside antibiotics (Rosenberg *et al.*, 2000; Aires and Nikaido, 2005). The genetic organisation of AcrD is

different from other RND systems in that it is not encoded alongside its own periplasmic adaptor protein but requires AcrA for proper function (Aires and Nikaido, 2005). To assess the impact of loss of AcrA on the function of the AcrD efflux pump the MICs of various aminoglycoside antibiotics to L976 could be determined. The MIC of the aminoglycoside antibiotic kanamycin to L976 ($\Delta acrA$) has already been determined (Table 3.1) and was not significantly different from that to wild-type but using a panel of aminoglycoside antibiotics may reveal increased susceptibility. It is necessary to use the *acrA* mutant from which the *aph* gene (an aminoglycoside phosphotransferase) has been removed as this confers resistance to kanamycin and may affect susceptibility to other aminoglycoside antibiotics.

There are multiple reports in the literature of the role of RND systems in resistance to antimicrobial peptides (AMPs) (Shafer *et al.*, 1998; Tzeng *et al.*, 2005; Eswarappa *et al.*, 2008; Warner *et al.*, 2008). However there is conflicting evidence about the involvement of AcrAB-TolC of *E. coli* in the resistance to AMPs (Rieg *et al.*, 2009; Warner and Levy, 2009). To investigate the role of AcrAB-TolC in resistance of *S. Typhimurium* to AMPs the resistance of SL1344 and the efflux mutants to a variety of AMPs could be tested by MIC and by killing assays.

Work has begun to further investigate the lack of AcrA protein detected in the TolC mutant. The *tolC* gene has been inserted into the pBAD vector (Invitrogen) and transformed into L109, the *tolC* mutant. Transcription of the *tolC* gene is inducible by arabinose and inhibited by glucose. This will allow a number of experiments to be performed. Firstly, cells will be grown in the presence of arabinose, so that the *tolC* will be transcribed, and then arabinose will be removed by washing and glucose added, to inhibit the transcription of *tolC*. Samples

will be taken from the culture before switching to glucose, and at time points afterwards and the proteins isolated. Western blotting can then be performed to confirm the level of TolC expression and determine whether AcrA can be detected after expression of *tolC*/TolC is stopped. The experiment will also be performed in reverse; the culture will be grown first in the presence of glucose, inhibiting *tolC* transcription, and then switched to arabinose, allowing *tolC* transcription. Western blotting will again be used to confirm TolC levels and determine whether AcrA can be detected. If this technique proves useful, another possibility is to perform the experiment in a *degP* mutant. DegP is a protease which degrades misfolded proteins in the periplasm (Strauch and Beckwith, 1988) and therefore this may be informative as to whether AcrA is absent because it is degraded if the AcrAB-TolC complex is unable to form.

3.9 Key Findings

- The *acrA* gene of *S. Typhimurium* was inactivated by insertion of the *aph* gene. The mutation was transduced back into SL1344 using P22 phage, the *aph* gene was removed using pCP20 and the mutation was complemented.
- Reverse-transcriptase PCR confirmed that *acrB* was still transcribed in the *acrA* mutant. Furthermore, western blotting showed that the AcrB protein was still translated in the absence of AcrA.
- The growth of the strain lacking AcrA was not significantly different from SL1344 in aerobic conditions.

- Inactivation of *acrA* led to a two-fold greater accumulation of Hoechst H3342 indicating a lower level of efflux.
- Lack of AcrA resulted in hyper-susceptibility to a range of antimicrobials. The *acrA* mutant was more susceptible than the *acrB* mutant to ciprofloxacin, triclosan, acriflavine and fusidic acid. Although the *tolC* mutant was generally the most susceptible, the *acrA* mutant was more susceptible than the *tolC* mutant to ciprofloxacin and triclosan.
- There was no difference between the size or appearance of the *acrA* mutant when compared to SL1344 using electron microscopy.

Chapter Four

The Role of AcrAB-TolC in infection

4. The Role of AcrAB-TolC in infection

4.1 Background

Previous work in this laboratory showed that *S. Typhimurium* mutants lacking either AcrB or TolC were less able to infect tissue culture cells. An AcrB mutant had a reduced ability to invade human embryonic intestinal cells (INT-407) and murine macrophages (RAW 264.7) while mutants lacking TolC had a reduced capacity to both adhere to and invade INT-407 cells and additionally were unable to invade murine macrophages (RAW264.7). Electron microscopy revealed that the *acrB* and *tolC* mutants induced fewer membrane ruffles on the surface of infected INT-407 cells. Furthermore, *in vivo* experiments in the avian host showed that colonisation and persistence were significantly reduced in mutants lacking either AcrB or TolC (Buckley *et al.*, 2006). Coupled with data from Nishino *et al.*, (2006) showing that AcrAB and TolC mutants were attenuated in the mouse model of *S. Typhimurium* infection this suggests that the AcrB and TolC proteins are needed for adhesion and invasion of *S. Typhimurium* and therefore have an important role in virulence.

4.2 Aims and Hypotheses

The initial aim of this section of work was to determine the effect of loss of *acrA* on the ability of *S. Typhimurium* to infect tissue culture cells. It was shown in chapter 3 using the fluorescent dye Hoechst 33342 that loss of any component of this efflux system impaired efflux function (Section 3.6.2). It was therefore hypothesised that this defect would translate into an impaired ability to adhere to and invade eukaryotic cells *in vitro*, as seen for mutants lacking AcrB or TolC. Subsequent work then used confocal imaging of SL1344 and strains

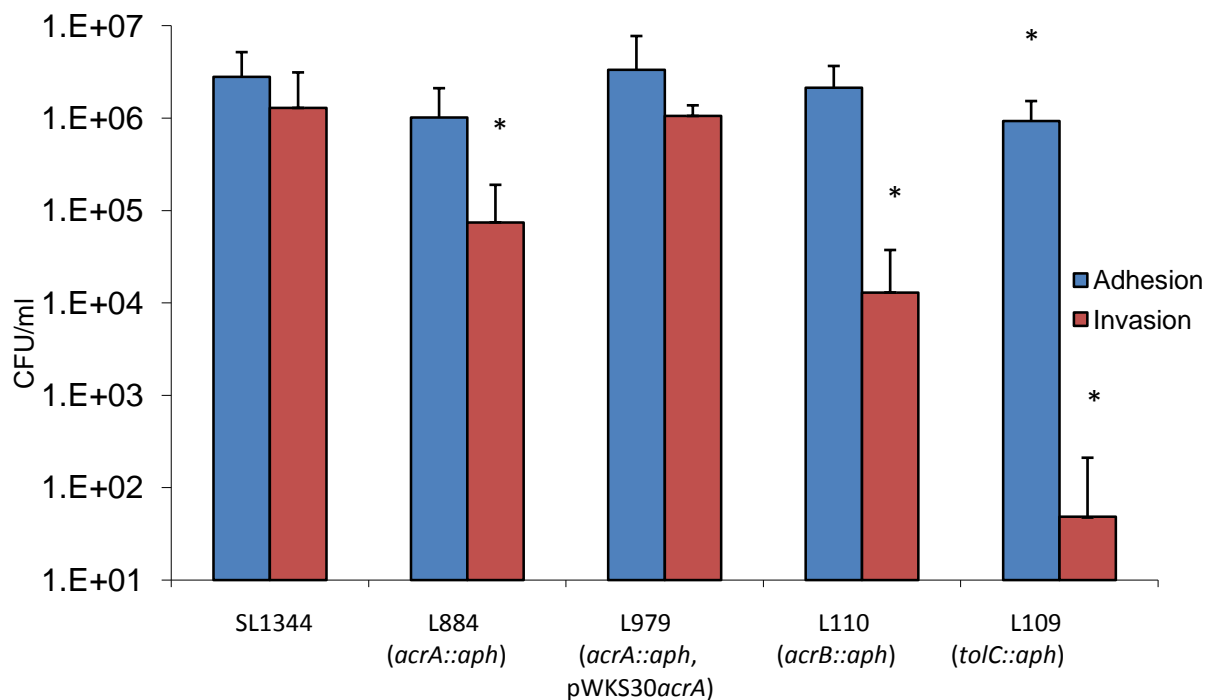
lacking AcrA, AcrB or TolC while infecting INT-407 cells to see if the infection patterns were variant between the mutants. A secondary aim in this section was to characterise the effect of growth in different media on the ability of Salmonella to infect INT-407 and whether this influenced the ability of the efflux mutants to infect tissue culture cells.

4.3 The effect of inactivation of *acrA* on the ability of *S. Typhimurium* to infect tissue culture cells

The abilities of *S. Typhimurium* SL1344 and the *acrA*, *acrB* and *tolC* efflux mutants (L884, L110 and L109) to adhere to and invade human embryonic intestine cells (INT-407) and mouse macrophages (RAW 264.7) were determined in the same experiments in parallel.

The *acrA* mutant (L884, *acrA::aph*) had a significantly reduced ability to invade INT-407 cells compared to SL1344 (11 % of SL1344 invasion, $P = 0.0009$). However, adhesion was not significantly altered by inactivation of *acrA* (84.4 % of SL1344 adhesion, $P = 0.33$) (Figure 4.1 a and b). The levels of adhesion and invasion in the strain in which *acrA* had been complemented with pWKS30*acrA* were not significantly different from SL1344 levels (119.8% of SL1344 adhesion, $P = 0.71$ and 81.88% of SL1344 invasion, $P = 0.47$). As previously published (Buckley *et al.*, 2006) the ability of the *acrB* mutant (L110, *acrB::aph*) to adhere to the INT-407 cells was not significantly changed (76.4% of SL1344 adhesion, $P = 0.06$). Again, as found by Buckley *et al.*, (2006) the *acrB* mutant had a reduced ability to invade the INT-407 cell line; this attenuation was greater than that seen upon inactivation of *acrA* (1.004% of SL1344 invasion, $P = 0.0002$). As previously shown (Buckley *et al.*, 2006), the *tolC* mutant (L109, *tolC::aph*) was the most attenuated. The level of adhesion was only 33.4% of

Figure 4.1 a. The ability of *S. Typhimurium* SL1344 and various efflux mutants thereof to adhere to and invade human embryonic intestine cells (INT-407).



Data are displayed as mean of at least three separate experiments performed in triplicate + standard deviation. Values returning a p value of ≤ 0.05 from a Student's T-test comparing mutants to the SL1344 values are denoted by *.

b. Adhesion and Invasion as a % of SL1344 values

	Adhesion (% of SL1344 value)	Invasion (% of SL1344 value)
SL1344	100	100
L884 (<i>acrA::aph</i>)	84.4 +/- 22.8	11.00 +/- 2.8
L979 (<i>acrA::aph</i>, pWKS30<i>acrA</i>)	119.8 +/- 23.4	81.88 +/- 24.8
L110 (<i>acrB::aph</i>)	76.4 +/- 18.2	1.004 +/- 0.09
L109 (<i>tolC::aph</i>)	33.4 +/- 11.3	0.003 +/- 0.012

Data are presented +/- standard deviation. Bold values indicate those that are significantly different to those for SL1344

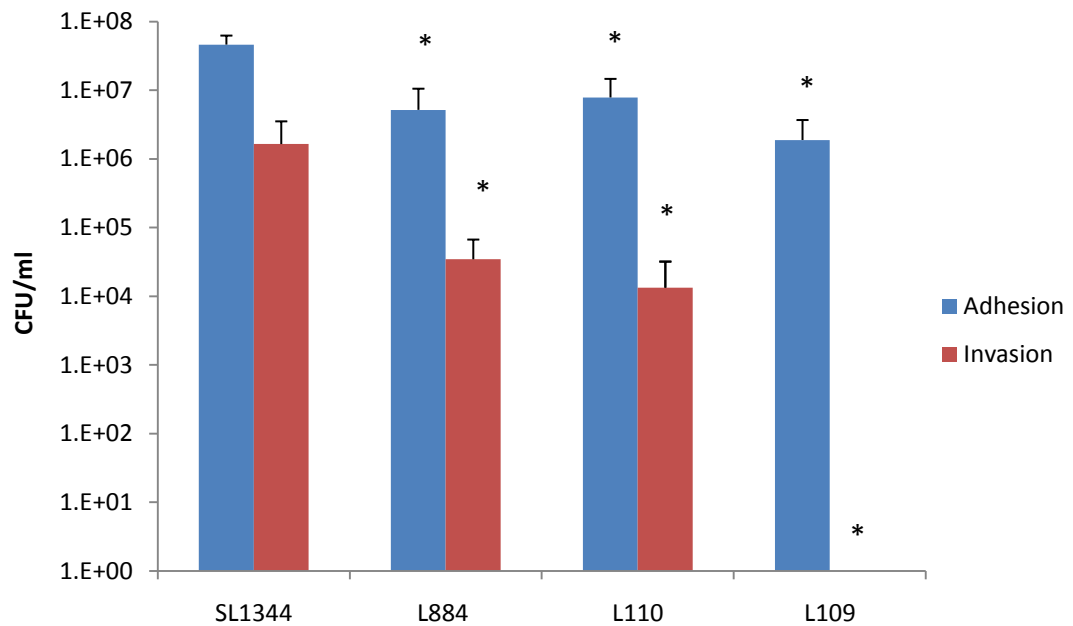
the SL1344 level ($P = 0.0002$) and the level of invasion was only 0.003% of the SL1344 level ($P = 0.0002$).

In the mouse macrophage cell line RAW 264.7 there was a slightly different pattern of attenuation. The *acrA* mutant had a significantly reduced ability to adhere to and invade the macrophages (11.22% of SL1344 adhesion, $P = 0.030$ and 2.1% of SL1344 invasion, $P = 0.01$) (Figure 4.2). The level of adhesion and invasion in the *acrB* mutant was not significantly different from *acrA* mutant (17.01% of SL1344 adhesion and 0.81% of SL1344 invasion). L109 (*tolC::aph*) had an impaired ability to adhere to RAW264.7 cells and was unable to invade (4.08% of SL1344 adhesion, $P = 0.002$ and 0% SL1344 invasion, $P = 0.00001$). Data in figure 4.2 was obtained in year one of the PhD programme before the complemented strain and transduced strain had been constructed. The experiments were repeated in year three by an undergraduate project student and included the transduced strain (L976) and the complemented strain (L979) (Appendix 2). The percentage attenuation of L884 was similar between the two data sets (14.8% SL1344 adhesion and 4.9% of SL1344 invasion). The level of adhesion and invasion by the *acrA* mutant from which the *aph* gene has been removed, L976 (Δ *acrA*), was not significantly different from the L884 levels ($P = 0.8$). Complementation of the *acrA* mutation with pWKS30*acrA* in the strain L979, ablated the mutant phenotype giving adhesion and invasion levels that were not significantly different from SL1344 ($P = 0.080$ and 0.055 , respectively).

4.4 Confidence Interval for level of SL1344 association

A large number of data sets (>30) for the association, adhesion and invasion levels for the control strain SL1344 with INT-407 cells was available from all replicates of this experiment

Figure 4.2 a. The ability of *S. Typhimurium* SL1344 and various efflux mutants thereof to adhere to and invade mouse macrophage cells (RAW 264.7).



Data are displayed as mean of at least three separate experiments performed in triplicate + standard deviation.

Values returning a p value of ≤ 0.05 from a Student's T-test comparing mutants to the SL1344 values are denoted by *.

b. Adhesion and Invasion as a % of SL1344 values

	Adhesion (% of SL1344 value)	Invasion (% of SL1344 value)
SL1344	100	100
L884 (<i>acrA::aph</i>)	11.22	2.1
L110 (<i>acrB::aph</i>)	17.01	0.81
L109 (<i>tolC::aph</i>)	4.08	0

Bold values indicate those that are significantly different to those for SL1344

and also from all subsequent tissue culture experiments as this infection was used as a control throughout. This allowed the range in which the CFU/ml for the association of SL1344 to be calculated and determination of whether this strain performed the same in each experiment. The interval was only calculated for association for simplicity and also because the level of association encompasses both adhesion and invasion values. At the 0.05 level of significance the confidence interval for association of SL1344 to INT-407 cells was calculated to be 1.2×10^6 to 1.1×10^7 CFU/ml. Data from further experiments were only considered acceptable if the SL1344 association values fell within this range.

4.5 The Effect of Triton X-100 on Bacterial Survival

The non-ionic surfactant Triton X-100 was used in all the tissue culture assays to disrupt the eukaryotic cell monolayer allowing subsequent enumeration of the bacteria. As inactivation of AcrAB-TolC can confer increased susceptibility to detergents it was important to ensure that, under the conditions of the tissue culture experiment, this did not influence bacterial enumeration.

The MIC of an agent is determined after 18-24 hours incubation of bacteria and test agent and under these conditions the MIC of Triton X-100 for L884 and L110 was the same as for SL1344, >20,000 µg/ml (Table 3.1), which is almost double the concentration used in the tissue culture assays (10,700 µg/ml or 1%). However, for L109 the MIC of Triton X-100 was 1200 µg/ml. Nonetheless it was shown previously that 1% Triton X-100 (as used in the tissue culture assays) had no affect on viability of either the *acrB* or *tolC* mutants (L110 and L109, respectively) during the time-span of exposure (30 minutes) during the experiment (Buckley

et al., 2006). Experiments were carried out to ensure that the *acrA* mutant (L884) was not adversely affected by the detergent.

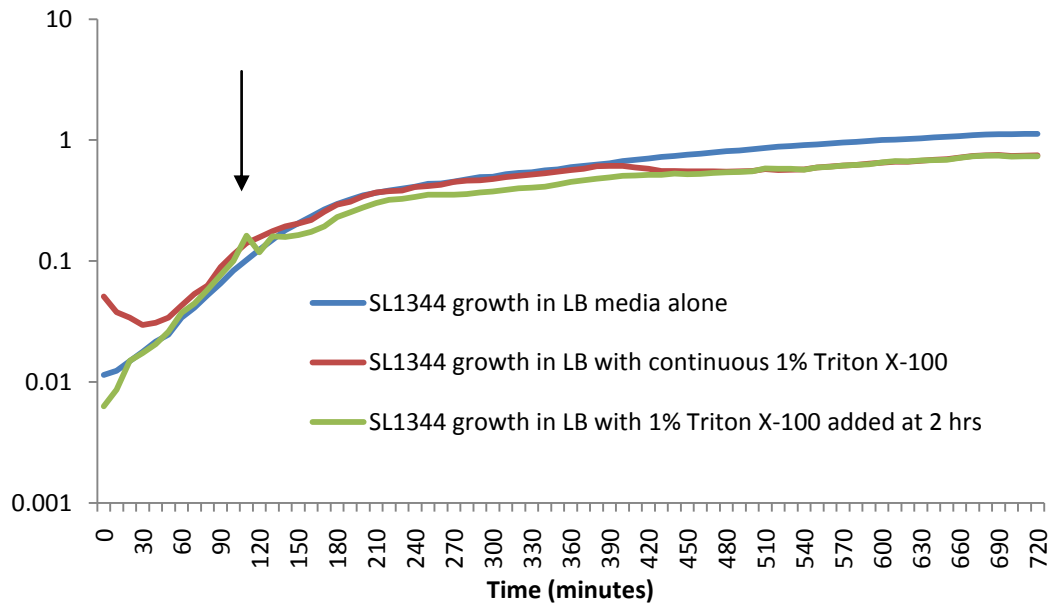
The growth of the *acrA* mutant was compared when grown in LB broth alone, in the continuous presence of 1% Triton X-100 and when 1% Triton X-100 was added after two hours growth (to replicate the tissue culture experiments). In this final test a visible drop in optical density (shown by the arrow in Figure 4.3) can be seen at the two hour mark. This was caused by the dilution effect of adding Triton-X-100 and the optical density recovers quickly. There was no significant difference between the generation times of SL1344 and L884 (*acrA::aph*) grown in LB broth, whether in the continuous presence of 1% Triton X-100 or when Triton X-100 was added after two hours growth (Figure 4.3).

4.6 Confocal microscopy images of INT-407 cells infected with SL1344 or mutants lacking AcrA, AcrB or TolC

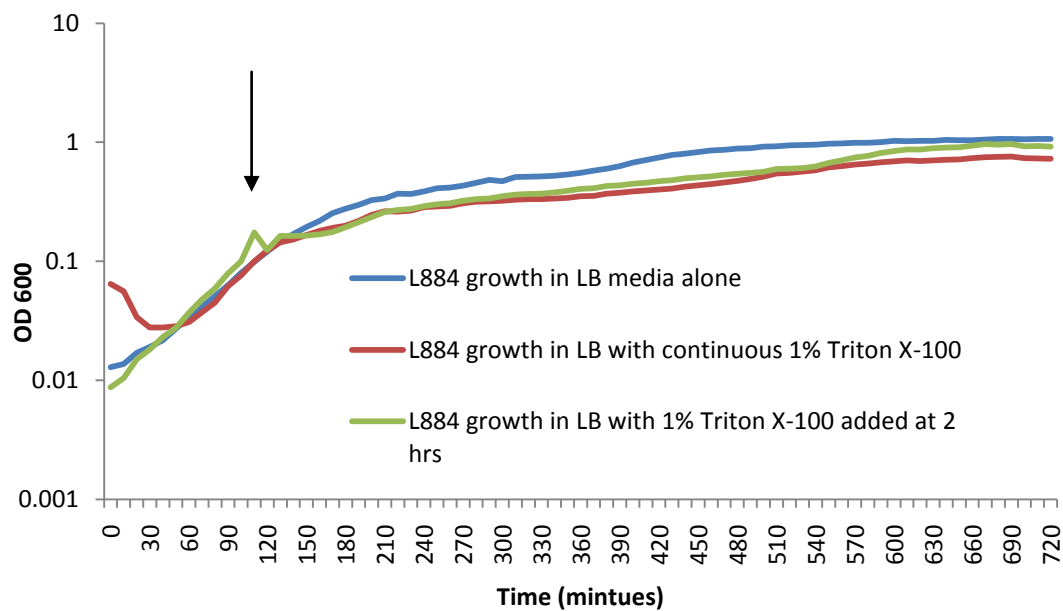
Confocal imaging was used to visualise infections of INT-407 cells with SL1344 or strains lacking AcrA, AcrB or TolC after growth in LB media to see if the effect of loss of the efflux pump genes visibly affected the infection pattern on the monolayer. SL1344 and strains with either *acrA*, *acrB* or *tolC* inactivated were transformed with the pUA66pacpP plasmid (Neil Burton, University of Birmingham) encoding green fluorescent protein (GFP) to give the strains L1019, L1020, L1021 and L1022, respectively. INT-407 monolayers were infected with each the GFP expressing strains and after fixing the plates were stained red with the F-actin specific molecule phalloidin conjugated to the Rhodamine dye. Scanning confocal microscopy images were taken of each infection (Figure 4.5). Bacteria lacking either AcrB or TolC appeared clumped on the surface of the eukaryotic cells with large areas under the

Figure 4.3. Growth of SL1344 and L884 in the presence of Triton X-100

a. The growth SL1344 in LB broth alone or in the presence of 1% Triton X-100



b. The growth L884 (*acrA::aph*) in LB broth alone or in the presence of 1% Triton X-100

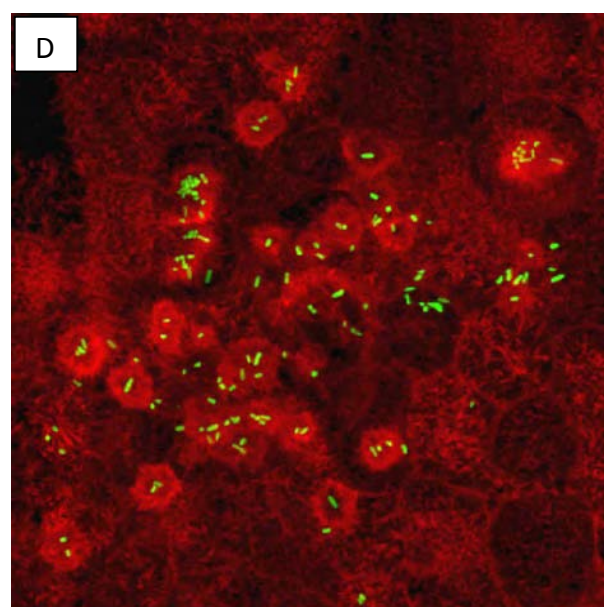
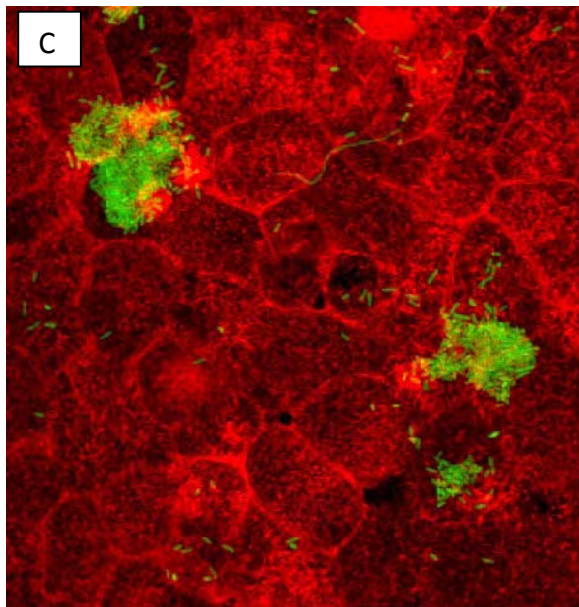
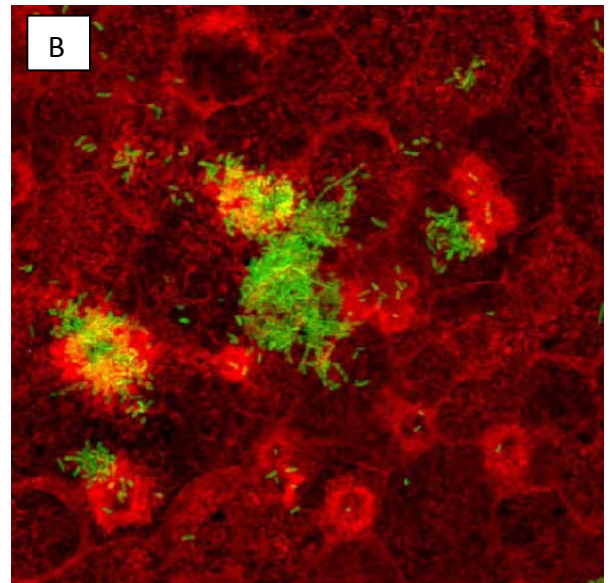
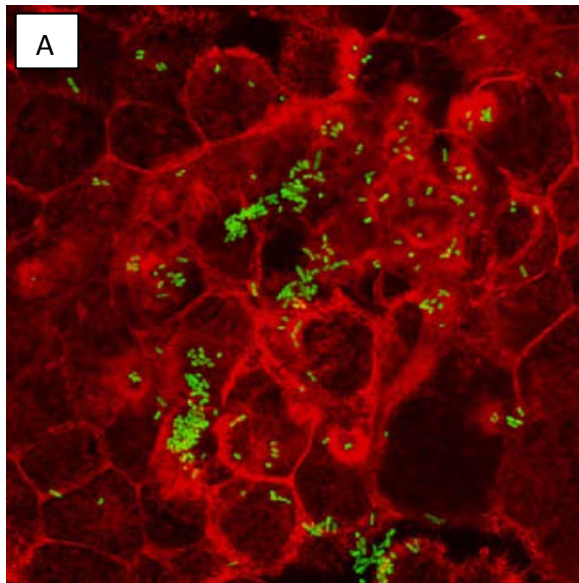


In each case the arrow indicates the point of addition of Triton X-100. Data is presented as the mean of three independent experiments.

Figure 4.4 Confocal microscopy images of INT-407 cells infected with wild-type and efflux mutant strains containing the GFP encoding pUA66*pacpP* plasmid.

Images taken by Dr. Rob Shaw (University of Birmingham). Eukaryotic cells are stained with the F-actin specific molecule phalloidin conjugated to the Rhodamine dye.

- A. L1019 = SL1344 with pUA66*pacpP*
- B. L1020 = L644 (Δ *acrB*) with pUA66*pacpP*
- C. L1021 = L742 (Δ *tolC*) with pUA66*pacpP*
- D. L1022 = L976 (Δ *acrA*) with pUA66*pacpP*



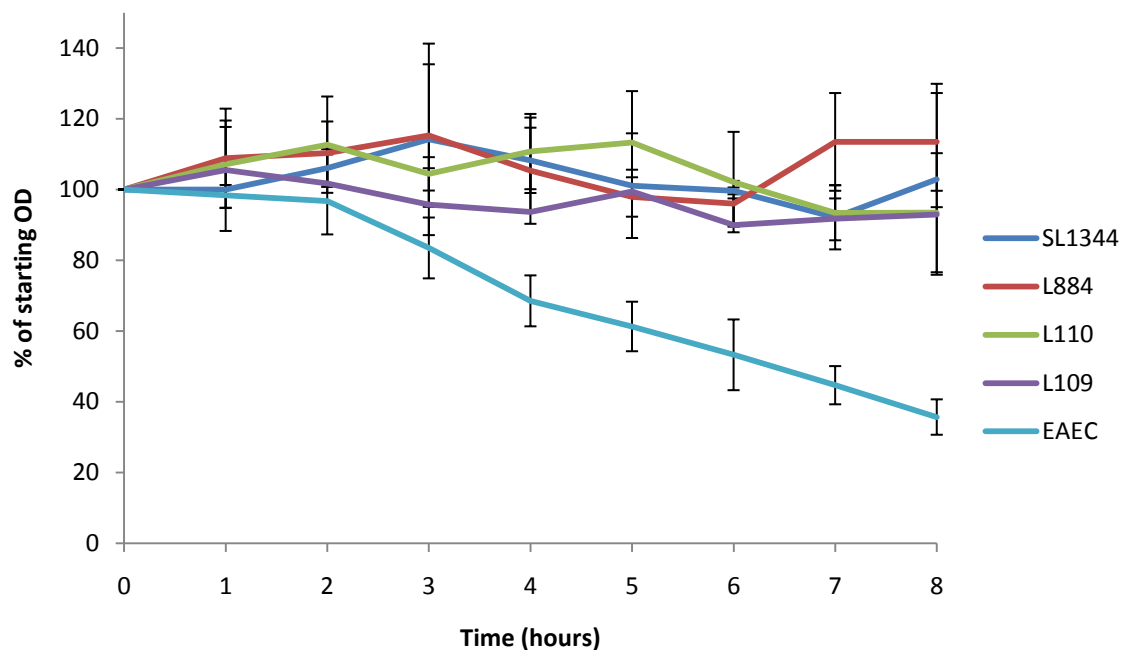
clumps staining bright red, indicating actin rearrangement (Figure 4.5 B and C, respectively). However, SL1344 and the strain lacking AcrA were evenly spaced over the surface of the monolayer and high actin activity was seen around single, or small numbers, of bacteria (Figure 4.5 A and D).

Experiments were performed to determine whether L110 and L109 aggregated in broth culture or whether the aggregation only occurred when the mutants came into contact with tissue culture cells. SL1344 and the three efflux mutants were grown overnight in broth culture and then left to stand for eight hours. Samples were taken from the surface of the culture every hour and the optical density measured. Strains which aggregate settle faster in broth, causing the optical density at the surface of the culture to clear more quickly. There was no significant difference between the optical density of any of the strains at the beginning of the experiment and after 8 hours (Figure 4.5). This suggests that neither SL1344 nor the efflux mutants aggregate in broth culture. Therefore, the aggregation seen by L110 and L109 in Figure 4.4 is likely to be a result of interaction between the bacteria and the eukaryotic cells.

4.7 Discussion

Inactivation of the gene encoding the periplasmic adaptor protein, *acrA*, significantly reduced the ability of *Salmonella* Typhimurium to invade INT-407 cells and to adhere to and invade RAW 264.7 cells compared with the parental strain, SL1344. As previously published, inactivation of either *acrB* or *tolC* also led to a reduction in the invasive ability of each mutant compared with the parental strain and inactivation of *tolC* also affected adherence (Buckley *et al.*, 2006).

Figure 4.5 Experiment to determine whether strains aggregate in broth culture.



Strain	Mean Final OD as % of starting OD +/- SD	T-test (<i>P</i>)
SL1344	102.9 +/- 24.3	0.42
L884	113.47 +/- 13.8	0.083
L110	93.46 +/- 16.83	0.26
L109	92.95 +/- 2.07	0.3
EAEC	35.70 +/- 5.0	0.03

Strains were grown overnight in LB broth and then left stationary for eight hours. The optical density at 600nm was measured every hour. Data are presented as a percentage of the starting OD value for each strain +/- the standard deviation. Data shown are the mean of three independent experiments.

This method of measuring adhesion and invasion is based on an established and widely used technique (for example Dibb-Fuller *et al.*, 1999) and clearly showed a difference in phenotype between SL1344 and the efflux mutants. However, there are a number of caveats to interpretation of data obtained with this technique. Firstly, it is not possible to distinguish between a defect in invasion versus a defect in intracellular survival as these would both present as a decrease in the number of bacteria after lysis of the eukaryotic cells. To more accurately distinguish between invasion/internalisation versus intracellular survival a time course experiment should be carried out in which the number of internal bacteria is enumerated at different time points after infection. In this experiment an invasion defect would result in low numbers of internal bacteria throughout the time course while a strain with a survival defect would have normal initial invasion levels which decreased significantly over the course of the experiment as the bacteria would be unable to survive in the intracellular environment.

The number of adhered bacteria could be complicated by bacterial adherence to the plastic. The effect of this could be tested by performing a mock assay in wells which do not contain eukaryotic cells. This will confirm whether the washing steps are sufficient and whether adhesion of bacteria to the plastic is affecting the measured level of adhesion. Furthermore, microscopy techniques could be employed to enumerate the number of bacteria adhered to the plastic and to the eukaryotic cells. To further investigate, and more accurately measure, the level of adhesion of bacteria to the eukaryotic cell surface internalisation could be blocked either by the use of cytochalasin B to block phagocytosis (Finlay *et al.*, 1991) or by the use of an *invA* mutant that is completely defective for invasion of cultured epithelial cells (Galan *et al.*, 1992). In this way no bacteria should enter the eukaryotic cells and the only

bacteria recovered from the assay will be those which have adhered. This approach will also allow confirmation that the gentamicin step kills all external bacteria.

Lastly, it is possible that as the assay was started with a large initial inoculum and then incubated for two hours at 37 °C the bacteria could have been growing in the tissue culture medium. However, if the mutants are less able to grow in the tissue culture medium than SL1344, this could skew the results, amplifying the apparent adhesion or invasion defect. Therefore, the growth kinetics of all strains in the different types of media (MEM and DMEM) should be studied.

In this study the effect of 1% Triton X-100 on bacterial enumeration was ruled out as a factor in the observed attenuation of the efflux mutants over the relevant time period. However, the effect of Triton X-100 was tested upon bacterial cells which had been grown overnight in LB broth whereas, in the subsequent tissue culture assays, the bacteria had been grown overnight in LB broth and then maintained in tissue culture medium for at least two hours before exposure to Triton X-100. While this is a notable inconsistency in the methodology of this study, it is important to note that in a previous study from this laboratory tests were carried out that simulated the association and invasion assays more precisely and exposure to 1% Triton X-100 had no significant effect on bacterial enumeration of the *acrB* or *tolC* mutants (Buckley *et al.*, 2006).

In chapter three, it was shown that inactivation of *acrA* affected antimicrobial susceptibility and efflux levels, and these data suggest that the PAP is also important for the virulence-related phenotypes of adhesion and invasion. This fits well with the growing body of evidence showing that RND systems of many bacterial species are required for virulence. For

example mutants lacking either *acrB* or *tolC* were less able to colonise and persist in the avian gut (Buckley *et al.*, 2006) and Nishino and colleagues reported that *S. Typhimurium* lacking AcrAB-TolC had attenuated virulence in the mouse model (Nishino *et al.*, 2006). Other examples of the involvement of homologues of AcrAB-TolC in virulence include the finding that AcrAB-TolC of the plant pathogen *Erwinia amylovora* is needed for virulence (Burse *et al.*, 2004), MtrCDE was required for survival of *Neisseria gonorrhoeae* in the female mouse genital tract model (Jerse *et al.*, 2003), MexAB-OprM mutants of *P. aeruginosa* had a reduced ability to invade Madin-Darby Canine Kidney (MDCK) cells (Hirakata *et al.*, 2002) and *C. jejuni* lacking the CmeABC efflux pump was unable to colonise chickens (Lin *et al.*, 2003). Collectively, these data provide compelling evidence that these efflux systems are vital to the pathogenicity of a range of Gram-negative bacteria. However, this is the first study in which the effect of loss of the PAP alone on virulence-related phenotypes has been studied. Data presented herein shows that the PAP has its own role in adhesion and invasion and that the mutant phenotype is similar, but distinct, from the mutant phenotypes of strains lacking either of the other two pump components.

In the avian model the contribution of these efflux pump proteins to virulence can be partially explained because AcrAB-TolC and its homologues are involved in the transport of multiple host derived substrates such as bile and antimicrobial peptides, allowing survival in the hostile host environment (Warner and Levy, 2009). However, the transport of these host derived substrates cannot explain the attenuation seen in tissue culture experiments. Another explanation of the attenuation of mutants lacking components of AcrAB-TolC is the numerous changes in expression of genes involved in pathogenicity that have been reported previously by this laboratory (Webber *et al.*, 2009). For example, inactivation of *acrA* was

associated with lower expression of genes found in SPI-2 while inactivation of *acrB* or *tolC* caused reduced expression of genes in SPI-1 and a subsequent decrease in the exported SPI-1 effector SipA, SipB and SipC. Furthermore, other changes included reduced expression of genes involved in chemotaxis and motility after inactivation of *acrB* or *tolC*, and in the *acrB* mutant this was associated with diminished motility. Furthermore, after inactivation of *acrA* or *acrB* repression of the *nap* and *nir* operons were seen which adversely affected growth in anaerobic conditions compared to the parental strain, SL1344 (Webber *et al.*, 2009).

Other explanations for the involvement of AcrAB-TolC in virulence and virulence related phenotypes include the possibility that it is directly involved in the efflux of a virulence related factor which is exported less efficiently in the mutants incurring an infection defect. Alternatively it is possible that AcrAB-TolC exports some toxic molecule, possibly a metabolite, produced during infection and the inability to do this prevents the salmonella from causing infection. These hypotheses are explored further in chapter 5.

Visualisation of infections of INT-407 cells by GFP marked SL1344 and *acrA*, *acrB* and *tolC* mutants by confocal microscopy showed that the mutants have different infection patterns. Inactivation of *acrB* or *tolC* led to clumping of the bacteria on the eukaryotic cell surface while inactivation of *acrA* did not, giving an infection which looked much more like that of SL1344. It was shown that neither SL1344 nor any of the mutant strains aggregated in broth culture showing that this is a phenomena associated with infection only. It is postulated, that this could contribute to the loss of invasive ability as individual bacteria may be less able to induce their own uptake if they associate in aggregates. This finding is in contrast with work in Enteric-Aggregative *E. coli* (EAEC) which showed that TolC promoted aggregation as

inactivation of *tolC* caused less aggregation to occur both in broth culture and in Hep-2 adherence experiments (Imuta *et al.*, 2008).

There is evidence that commonly used plasmid vectors and also the presence of GFP, particularly when constitutively expressed, can inhibit virulence of *Salmonella enterica* in both tissue culture and in the *in vivo* mouse model (Knodler *et al.*, 2005; Clark *et al.*, 2009). Therefore, the presence of a plasmid encoded, constitutively expressed *gfp mut2* gene could be a contributing factor to the phenotypes seen. This is unlikely as the wild-type also contains the plasmid so could only be a factor if the efflux mutants are less able to cope with the added burden of maintaining the plasmid and expressing the fluorescent protein. Nonetheless, careful controls would need to be included in future experiments to ensure that any alteration in phenotype is not an artefact of the presence of the plasmid or GFP. These controls are discussed further in section 4.8.

4.8 Further work

As discussed in section 4.7, further experiments are required to validate the tissue culture technique and dissect out the effect of the gene inactivations on adhesion, invasion and intracellular survival. To differentiate between defects in invasion and intracellular survival time course assays will be performed in which the level of internal bacteria are enumerated at various time points after infection.

The infection assay should also be performed on wells that do not contain any eukaryotic cells. If no bacteria are recovered from these wells this will confirm that the washing technique is effective and that results are not being skewed by the differential ability of

different strains to adhere to the plastic. This could be further confirmed by microscopy to enumerate the number of bacteria adhered to the plastic and to the eukaryotic cells.

More accurate assessment of the level of adhesion should include blocking internalisation of bacteria into the eukaryotic cells. This could be achieved either by the use of the fungal metabolite cytochalasin B to block phagocytosis by inhibition of actin polymerisation or by the use of an *invA* mutant that is completely defective for invasion. Bacteria will then not be able to enter the eukaryotic cells and the only bacteria recovered from the assay will be those which have adhered. This approach along with MIC susceptibility testing would also allow confirmation that the gentamicin step kills all external bacteria. Finally, the growth of all strains should be measured in both MEM and DMEM to ensure that the attenuation discussed is not an artefact of a growth deficiency in these media.

To further investigate the effect of *acrA* inactivation on the ability to cause infection, experiments in the mouse and chicken models of infection, similar to those previously described for the *acrB* and *tolC* mutants, could be performed. The tissue culture data presented in this study suggests that the *acrA* mutant will be less attenuated than the *acrB* or *tolC* mutants.

It was suggested in the further work section of chapter 3 that a strain lacking all three components of the AcrAB-TolC efflux system and also strains which over-express the *acrA*, *acrB* and *tolC* genes could be constructed. The ability of these strains to adhere to and

invade tissue culture cells *in vitro* and cause infection in *in vivo* infection models could then be determined to learn more about the role of this efflux system in infection.

The aggregative phenotype of the *acrB* and *tolC* mutants should be confirmed by repeating the experiments with the inclusion of more stringent controls. Clark and colleagues (2009) reported that, while plasmid encoded GFP had an inhibitory effect on invasion of *Salmonella*, a chromosomally encoded version did not. Therefore, a better strategy would be to insert the *gfp* gene onto the chromosome and repeat the experiments. To check that there was no attenuation of invasive ability another infection assay should be run in parallel to the one to be fixed and visualised. In this assay the number of invaded bacteria should be enumerated as described previously and compared to the levels of each strain without the chromosomal *gfp*.

4.9 Key findings

- Inactivation of *acrA* reduced the ability of *Salmonella* Typhimurium SL1344 to invade INT-407 cells.
- Triton X-100 had no adverse affect on the growth of the *acrA* mutant therefore data from the tissue culture experiments are valid.
- Inactivation of *acrB* or *tolC* led to apparent bacterial clumping on the surface of INT-407 monolayers while inactivation of *acrA* seemed to have little effect on the distribution of the bacteria on the monolayer.

Chapter Five

**Does AcrAB-TolC Export A Molecule
Required For Virulence?**

Chapter 5 - Does AcrAB-TolC export a molecule required for virulence?

5.1 Background

Although the existence of a link between RND efflux pumps of many bacterial pathogens and virulence of the organisms is now well established, the reason behind this relationship is not fully understood. One hypothesis is that AcrAB-TolC exports a virulence related factor. This hypothesis is partly based on the observation by Hirakata *et al.*, (2002) that the addition of culture supernatant from MDCK cells infected with wild-type *Pseudomonas aeruginosa* K767 restored the ability of a *mexAB-oprM* (homologous to AcrAB-TolC) triple mutant (K1119) to invade MDCK cells. This suggests that the role of MexAB-OprM in virulence is mediated by the efflux of a virulence related factor.

5.2 Aims and Hypotheses

The initial aim was to recreate these experiments in *S. Typhimurium* to see whether a putative virulence related factor could be detected in culture supernatant. These experiments were performed with L109 (*tolC::aph*), which had the greatest attenuation of the *acrA*, *acrB* and *tolC* mutants to ascertain whether the ability to invade INT-407 cells *in vitro* could be restored by the addition of culture supernatant from SL1344 infected INT-407 monolayers.

In order to further explore the hypothesis that AcrAB-TolC exports a virulence related factor, experiments were performed to determine whether the ability of the efflux mutants L884 (*acrA::aph*), L110 (*acrB::aph*) or L109 (*tolC::aph*) to cause infection *in vitro* could be restored by co-infection with SL1344.

A related hypothesis was that AcrAB-TolC exports a eukaryotic cell-derived factor and that inactivation of components of the system prevent this transport, impairing the ability to cause infection *in vitro*. This was tested by addition of media conditioned by uninfected INT-407 cells to an infection of INT-407 cells with L109 (*tolC::aph*) to determine whether this could restore the invasive ability of the mutants.

The final hypothesis tested in this section was that AcrAB-TolC exports a bacterially derived factor which is required for invasion and that inactivation of components of the system prevent this transport which impairs the ability of the mutants to cause infection *in vitro*. This was tested by addition of media conditioned by growth of SL1344 to infections of INT-407 cells by the efflux mutants to determine whether this could restore the invasive ability of the mutants.

5.3 Can the virulence of the *tolC* mutant be restored by addition of supernatant from SL1344 infected INT-407 monolayers?

Firstly, the protocol of Hirakata *et al.*, (2002) was repeated, keeping as close to the original procedure as possible with alterations to certain parameters to make the protocol more suitable for investigation using Salmonella as the model organism (Figure 5.1). Instead of MDCK (canine kidney) cells INT-407 cells were chosen as the model as these are a human intestinal epithelial cell line and so they are more biologically relevant to salmonella infection. As a result of the choice of cell line, the eukaryotic cells were incubated for 48

Figure 5.1 Parameters tested while investigating restoration of invasion of L109 (*tolC::aph*) by addition of supernatant from an infection of INT-407 cells with SL1344. The Hirakata method below is taken from Hirakata *et al.*, (2002).

	Hirakata method	Parameters changed or explored
Cell Type	MDCK monolayers infected with wild-type <i>P. aeruginosa</i> (K767) and <i>mexAB-oprM</i> mutant (K1119)	INT-407 monolayers infected with wild type <i>Salmonella</i> (SL1344)
Growth phase of bacteria used in infection to create supernatant	Growth phase of bacteria not stated.	INT-407 cells infected with either stationary phase or log phase SL1344 to create supernatant.
Duration of incubation of eukaryotic cells with bacteria	Supernatant from this infection harvested after 3 hours	Supernatant harvested after 30 minutes, two hours or 18 hours (overnight).
Filter type	Supernatant filter sterilised (filter type not disclosed)	Supernatant filter sterilised with a low protein binding filter (0.2µm Millex®HV Durapore®)
Dilution of supernatant	Supernatant "diluted appropriately"	Supernatant diluted 1:2 and 1:20
Pre-incubation of eukaryotic cells with supernatant	Supernatant from infected MDCK cells added and pre-incubated with fresh MDCK cells for one hour before infection.	Supernatant from infected INT-407 cells added and pre-incubated for one hour before infection or added at time of infection with no pre-cubation.
Bacterial inoculum	Monolayers infected with 3.5×10^6 bacterial cells (K767 or K1119)	Monolayers infected with 1×10^7 bacterial cells (SL1344 or L109)
	Association and invasion assays performed as previously described.	

N.B. Only one parameter was changed in each experiment and each was performed in triplicate.

hours after seeding to achieve confluence rather than the 4 days required for MDCK cells. The bacterial inoculum for *Salmonella* infection was increased to 1×10^7 CFU/ml compared to the 3.5×10^6 CFU/ml for *P. aeruginosa* used by Hirakata (2002). This was so that a similar infective dose was used in these experiments as used previously during this study and previous studies carried out in this, and other, laboratories (Dibb-Fuller *et al.*, 1999; Buckley *et al.*, 2006). The test strain chosen for this investigation was L109 (*tolC::aph*) as this showed the greatest attenuation of associative, adhesive and invasive ability during this study and also in published data (Buckley *et al.*, 2006), theoretically making reduction in attenuation easier to detect.

However, no restoration of the invasive ability of the efflux mutant (L109, *tolC::aph*) was seen after addition of supernatant from INT-407 infected with SL1344. To determine whether this was a technical artefact alterations to various parameters were investigated (Figure 5.1).

The time that SL1344 was exposed to the tissue culture cells to provide the supernatant for testing was altered. Initially, the infection was allowed to proceed for only 30 minutes before the supernatant was harvested rather than the 3 hours used by Hirakata *et al.* This time scale was thought to be more biologically relevant as this is the time required for this strain of *S. Typhimurium* (SL1344) to adhere to and invade epithelial cells *in vitro* (La Ragione *et al.*, 2003). However, the numbers of SL1344 invading the INT-407 cell line was unchanged and L109 (*tolC::aph*) remained attenuated. Therefore, it was postulated that any exported factor may not be reaching a sufficiently high concentration to elicit any effect during this shorter incubation period. Therefore, the exposure time was increased to 2 hours and, in a separate

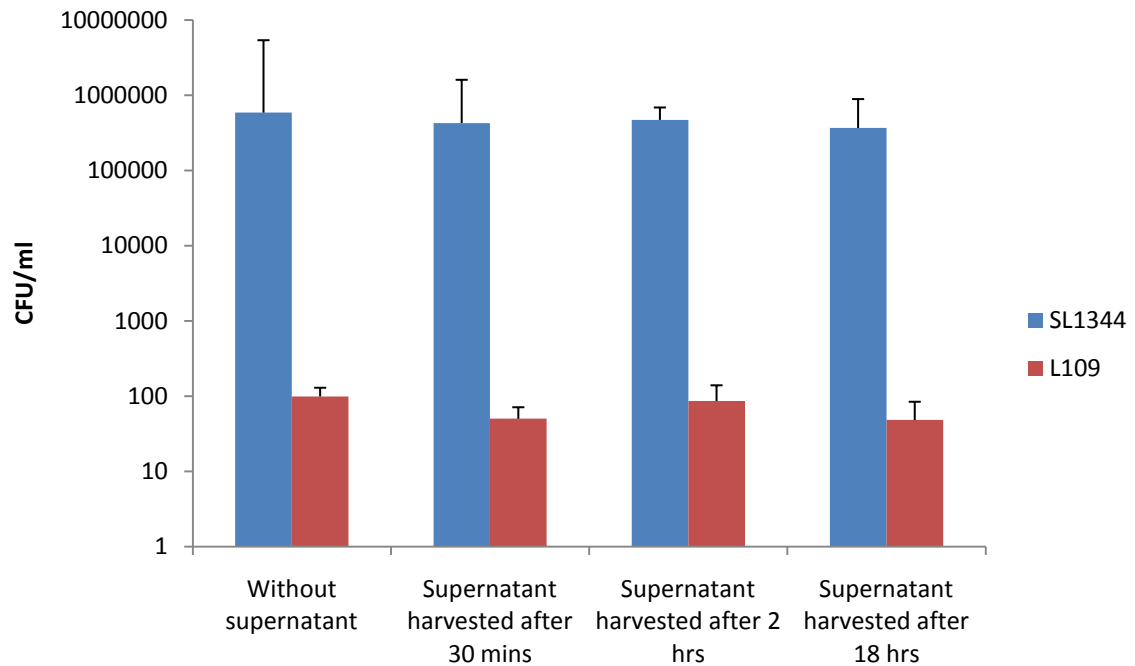
experiment, to 18 hours (overnight) thereby allowing higher concentrations of any exported factor to accumulate. However, the numbers of SL1344 invading was unchanged and L109 (*tolC::aph*) remained attenuated (Figure 5.2).

A bacterially derived factor may only be produced during a certain growth phase therefore supernatant was generated with either stationary phase (overnight) bacterial cultures or mid-logarithmic phase bacterial cultures (OD₆₀₀ of 0.6). The number of SL1344 invading was unchanged and L109 (*tolC::aph*) remained attenuated (Figure 5.3). As no difference was observed, stationary phase bacterial cultures were used in the remaining experiments to emulate those performed by Hirakata and colleagues (2002), earlier work during this study and other published work (Dibb-Fuller *et al.*, 1999; Buckley *et al.*, 2006).

The filter used in the experiments by Hirakata *et al.*, (2002) was not described in the publication and no reply to email correspondence was received. It was hypothesised that the filters used in earlier experiments with *Salmonella* may have bound and retained any putative exported virulence determinate. Therefore, low protein binding filters (0.2µm Millex®HV, Durapore® PVDF membrane) were used to ensure that any proteinaceous factor was not lost during filtration. However, this did not affect the outcome of the experiment as the number of SL1344 invading was unchanged and L109 (*tolC::aph*) remained attenuated (Figure 5.4).

The dilution factor for supernatants used in the study by Hirakata *et al.*, (2002) was also not specified in their publication and no reply to correspondence was received. Dilution of supernates 1:2 and 1:20 in fresh inoculation media were evaluated. The number of SL1344 invading was unchanged and L109 (*tolC::aph*) remained attenuated (Figure 5.5).

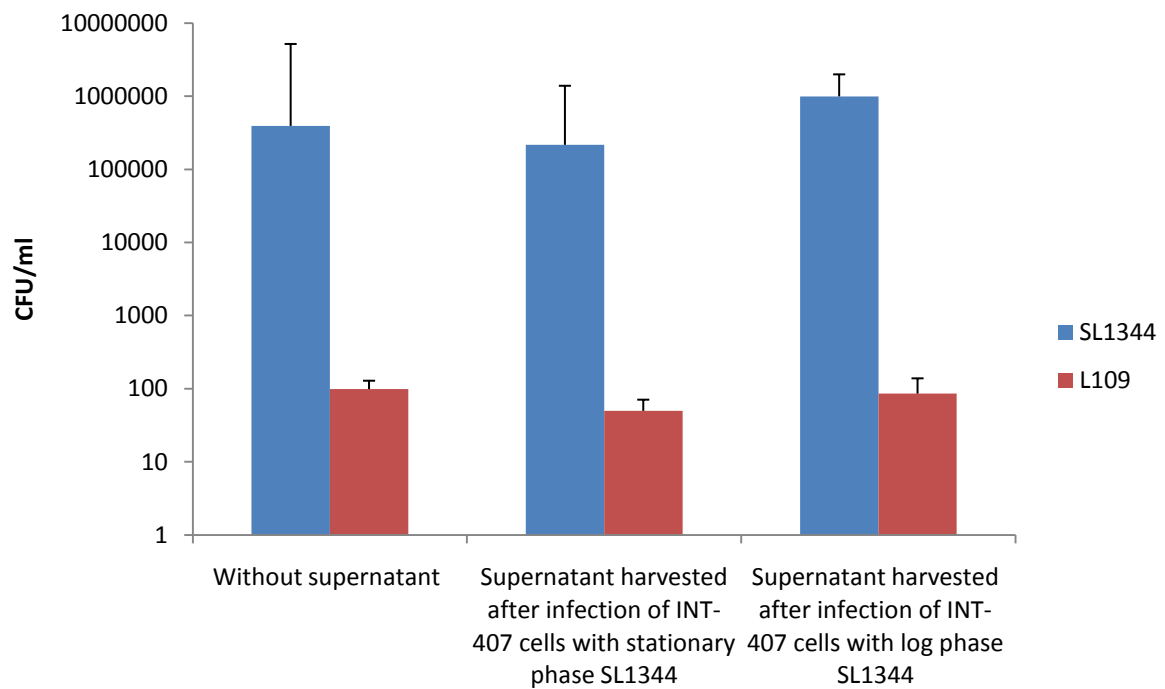
Figure 5.2 The effect of supernatant from SL1344 infected INT-407 cells on the ability of SL1344 and L109 (*tolC::aph*) to invade INT-407 cells: Supernatant harvested at 30 minutes, two hours or 18 hours post-infection.



To obtain the supernatant used in this experiment INT-407 cells were infected with SL1344 and the infection allowed to proceed for 30 minutes, two hours or 18 hours (overnight). The sterile supernatant from an infection of INT-407 cells with SL1344 was diluted 1:2 in inoculation media and used to dilute the bacterial suspension for the infection assay.

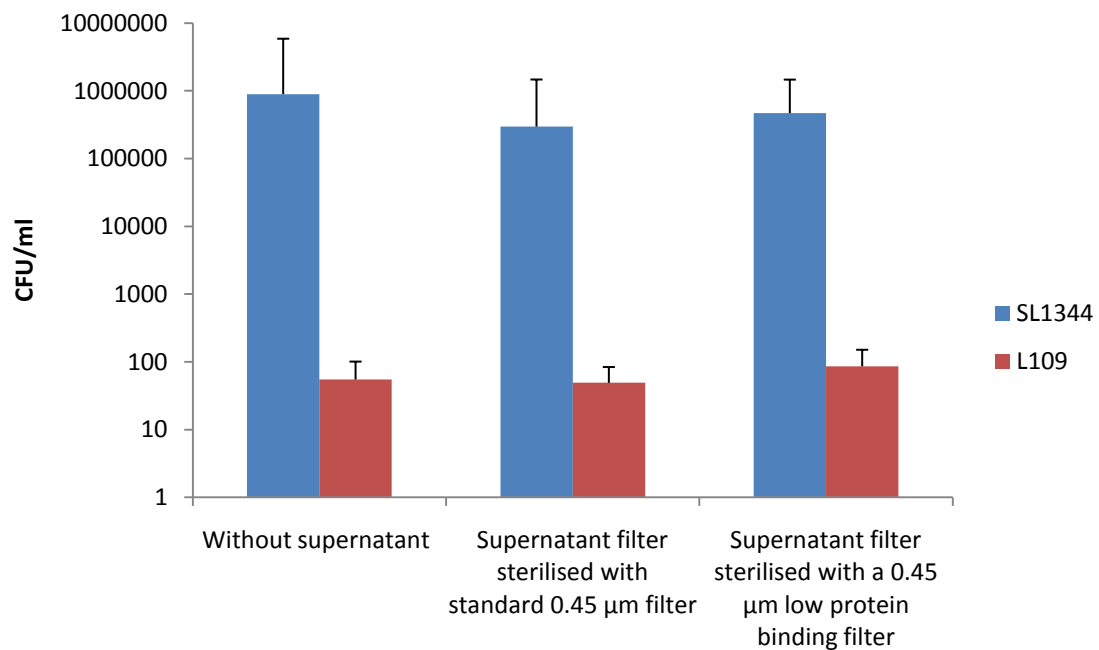
Data are displayed as the mean of three separate experiments performed in triplicate + standard deviation.

Figure 5.3 The effect of supernatant from SL1344 infected INT-407 cells on the ability of SL1344 and L109 (*tolC::aph*) to invade INT-407 cells: Supernatant taken from INT-407 cells infected with either stationary phase or log phase SL1344



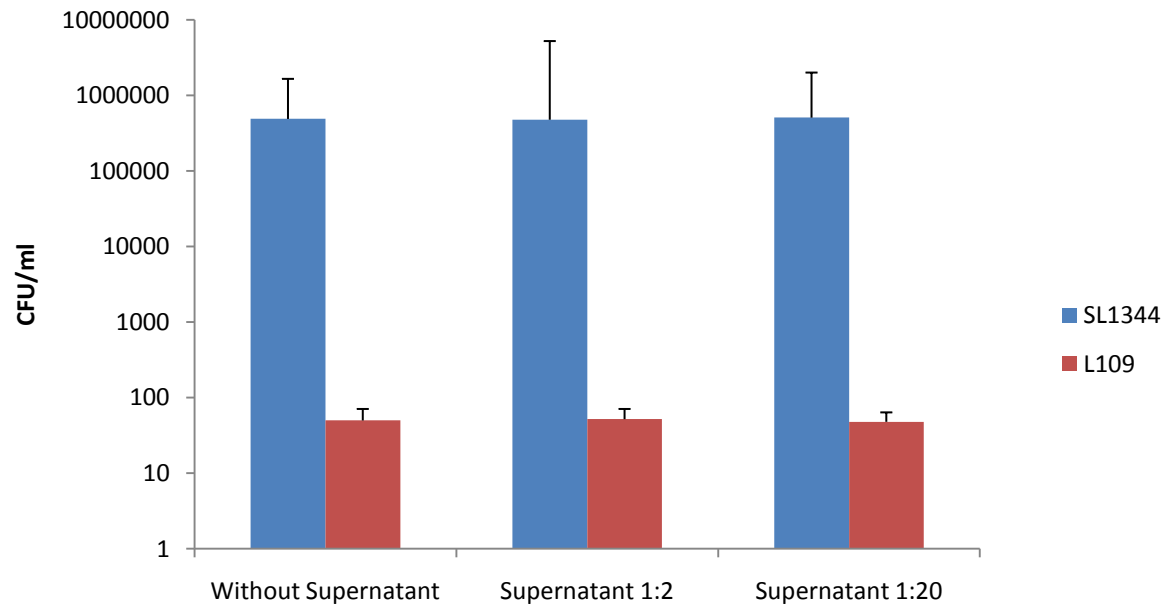
Supernatant was harvested from INT-407 cells which were infected with either stationary phase SL1344 (grown overnight) or log phase SL1344 (grown for ~ 2 hrs until OD₆₀₀ of 0.6). During this experiment supernatant was harvested after 2 hours and filter sterilised using a low protein binding filter (0.2µm Millex®HV Durapore® PVDF membrane). The sterile supernatant was diluted either 1:2 in inoculation media and used to dilute the bacterial suspension for the infection assay. Data are displayed as the mean of three separate experiments performed in triplicate + standard deviation.

Figure 5.4 The effect of supernatant from SL1344 infected INT-407 cells on the ability of SL1344 and L109 (*tolC::aph*) to invade INT-407 cells: Supernatant sterilised with different filter



During this experiment supernatant from an infection of INT-407 cells with SL1344 was harvested after 2 hours and filter sterilised with either a Millex[®] GP 0.45µm filter unit or a low protein binding filter (0.45µm Millex[®] HV Durapore[®] PVDF membrane). The sterile supernatant was diluted 1:2 in inoculation media and used to dilute the bacterial suspension for the infection assay. Data are displayed as the mean of three separate experiments performed in triplicate + standard deviation.

Figure 5.5 The effect of supernatant from SL1344 infected INT-407 cells on the ability of SL1344 and L109 (*tolC::aph*) to invade INT-407 cells: Supernatant diluted 1:2 or 1:20.



The sterile supernatant from an infection of INT-407 cells with SL1344 was diluted either 1:2 or 1:20 in inoculation media and used to dilute the bacterial suspension for the infection assay. During this experiment supernatant was harvested after 2 hours and filter sterilised using a low protein binding filter (0.2µm Millex®HV Durapore® PVDF membrane).

Data are displayed as the mean of three separate experiments performed in triplicate + standard deviation.

Once supernatant had been collected and diluted, Hirakata and colleagues added this to their monolayers and pre-incubated this for one hour before infection with their test strains. In the experiment with *Salmonella*, the assay was performed both with and without this pre-incubation step but the number of SL1344 invading was unchanged and L109 (*tolC::aph*) remained attenuated (Figure 5.6).

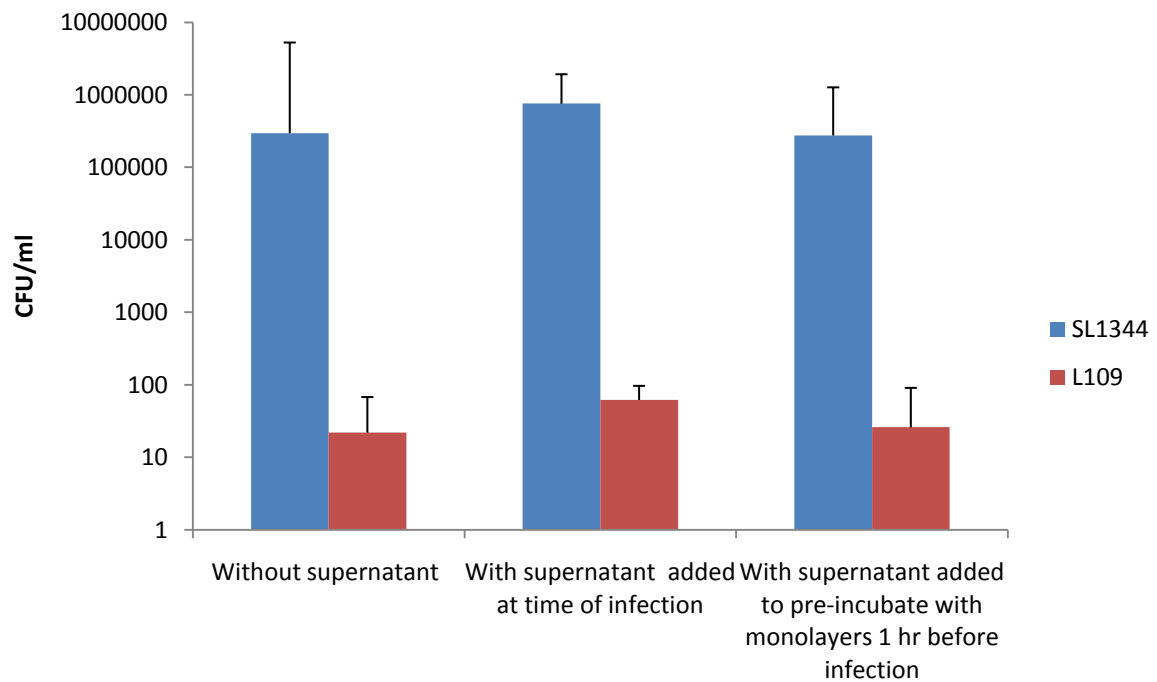
None of the variations of the experiment resulted in restoration of the invasive abilities of L109 (*tolC::aph*) or any change in the invasion of SL1344 (Figure 5.2-5.6).

5.4 Can the ability of the *acrA*, *acrB* and *tolC* mutants to adhere to and invade INT-407 cells be restored by co-infection with SL1344?

To further investigate the hypothesis that AcrAB-TolC exports a virulence related factor, INT-407 cells were infected with SL1344 in combination with each of the efflux mutants. The hypothesis was that, if a virulence related factor was exported by AcrAB-TolC, the invasive ability of the efflux mutants may be restored by co-infection because the factor would be provided by the wild-type.

In addition, these experiments were informative about the competitive fitness of SL1344 and the efflux mutants. Previous work with the *acrB* and *tolC* mutants included investigation of the ability of the mutants to compete with wild-type *Salmonella* and other gut flora in the poultry model of infection (Buckley *et al.*, 2006). In the one day old chick model the *tolC* mutant was unable to colonise and both the *acrB* and *tolC* mutants were unable to persist. In the two week old

Figure 5.6 The effect of supernatant from SL1344 infected INT-407 cells on the ability of SL1344 and L109 (*tolC::aph*) to invade INT-407 cells: Supernatant added at time of infection or pre-incubated with un-infected monolayers for 1 hour before infection with test strains.



During this experiment supernatant was harvested after 2 hours, filter sterilised using a low protein binding filter (0.2µm Millex®HV Durapore® PVDF membrane) and diluted 1:2 in inoculation media. This sterile supernatant was either used to dilute the bacterial suspension for the infection assay and therefore added at the time of infection or was added to the INT-407 monolayers, to be used in the infection assay, and incubated for one hour before infection with SL1344 and L109.

Data are displayed as the mean of three separate experiments performed in triplicate + standard deviation.

chick model, in which a gut flora is present, only the *acrB* mutant was tested; again this could colonise but not persist in the avian gastrointestinal tract (Buckley *et al.*, 2006). However, for this project on AcrA, the poultry model was not available so equivalent experiments were conducted in the tissue culture model to see how well the mutants could infect INT-407 cells when in competition with SL1344.

To establish the ability of each mutant to adhere to and invade INT-407 cells when in direct competition with the parental strain, SL1344, experiments were performed in which equal numbers of SL1344 and one of the mutants (L884, L109 or L110) were used to infect INT-407 cell monolayers. Viable counts were performed on the initial inoculum with replica plating used to distinguish the number of SL1344 from the kanamycin resistant mutant. In each experiment there was no significant difference between the number of SL1344 and mutant in the starting inoculum (Table 5.1). Infections with SL1344, L884, L109 or L110 alone were also performed in parallel to ensure that the same pattern was seen as described previously and so that the level of adhesion and invasion could be compared between infection alone and infection in competition. When infecting monolayers alone, each of the mutants produced a similar pattern of attenuation as described in section 4.1 and Figure 4.1. As before data giving a *P* value of less than 0.05 from a Student's *t*-test were taken as significant. Significant data for single infections are the same as those seen on figure 4.1. To avoid over complication on figures 4.3 and 4.4 the significant values are not indicated.

Table 5.1 Viable counts of inoculum used in competition assays

	SL1344 CFU/ml	Mutant CFU/ml
SL1344	4.8×10^5	-
L884	-	4.9×10^5
L110	-	5.1×10^5
L109	-	4.7×10^5
SL1344 and L884	2.3×10^5	2.6×10^5
SL1344 and L110	2.4×10^5	2.6×10^5
SL1344 and L109	2.3×10^5	2.2×10^5

Bacterial counts determined by serial dilution and sub-culture onto LB agar. Mutant and wild-type counts distinguished by subsequent replica plating onto LB agar supplemented with 50µg/ml kanamycin.

5.4.1 SL1344 and L884 (*acrA::aph*)

Following inoculation of INT-407 cells with a 50:50 mixture of SL1344 and L884, there was no significant difference between the mean number of adherent SL1344 and L884 (*acrA::aph*) ($P = 0.27$) (Figure 5.7 a). Furthermore, the numbers of L884 (*acrA::aph*) cells per ml that adhered when INT-407 cells were infected with L884 alone were not significantly different from those when co-infected with SL1344 ($P = 0.10$) (Figure 5.7 a).

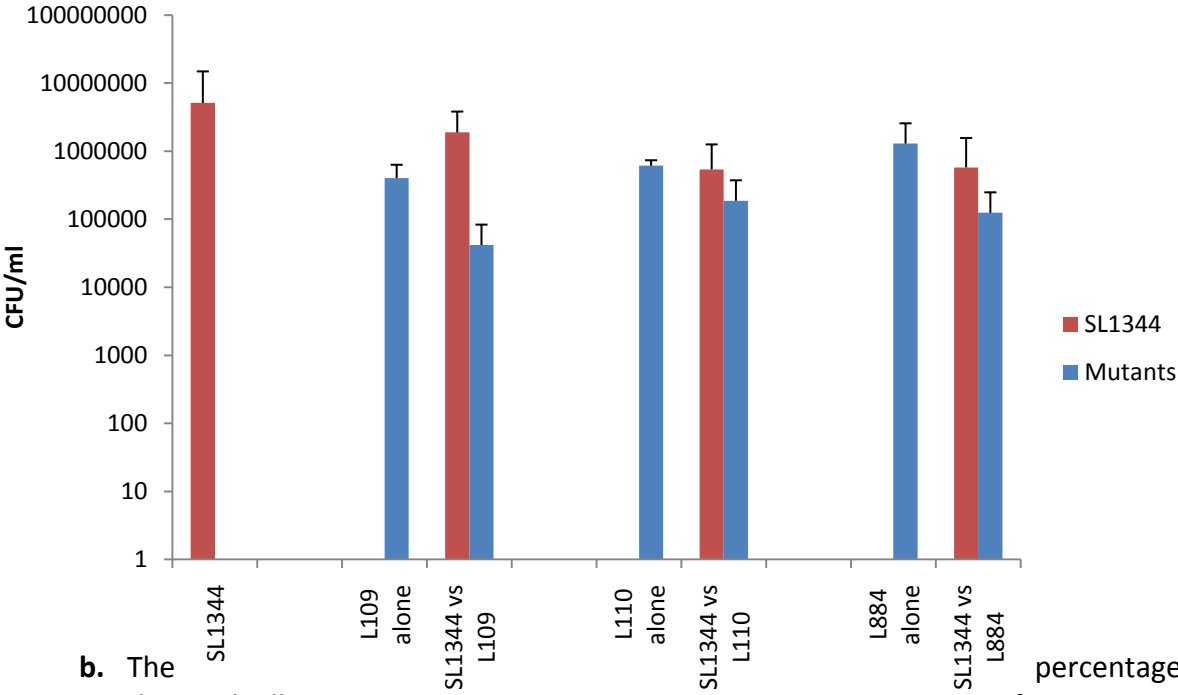
After infection with a mixture of SL1344 and L884, significantly more of the wild-type strain, SL1344, was able to invade the INT-407 cell line than L884 ($P = 0.024$) (Figure 5.8 a) with the mutant only accounting for 26.3% of the total invaded bacteria (Figure 5.8 b). However, the numbers of invaded L884 after mixed infection were not significantly different from the invasion level when L884 was inoculated alone, indicating that the difference in invasion level of the mutant was unaffected by the presence of SL1344. The numbers of SL1344 bacteria which adhered to the INT-407 cells were not significantly different to those when inoculated alone or with L884 ($P = 0.23$) (Figure 5.7 a). However, significantly fewer SL1344 were found to have invaded the INT-407 cells when inoculated in combination with L884 ($P = 0.03$) (Figure 5.8 a).

5.4.2 SL1344 and L110 (*acrB::aph*)

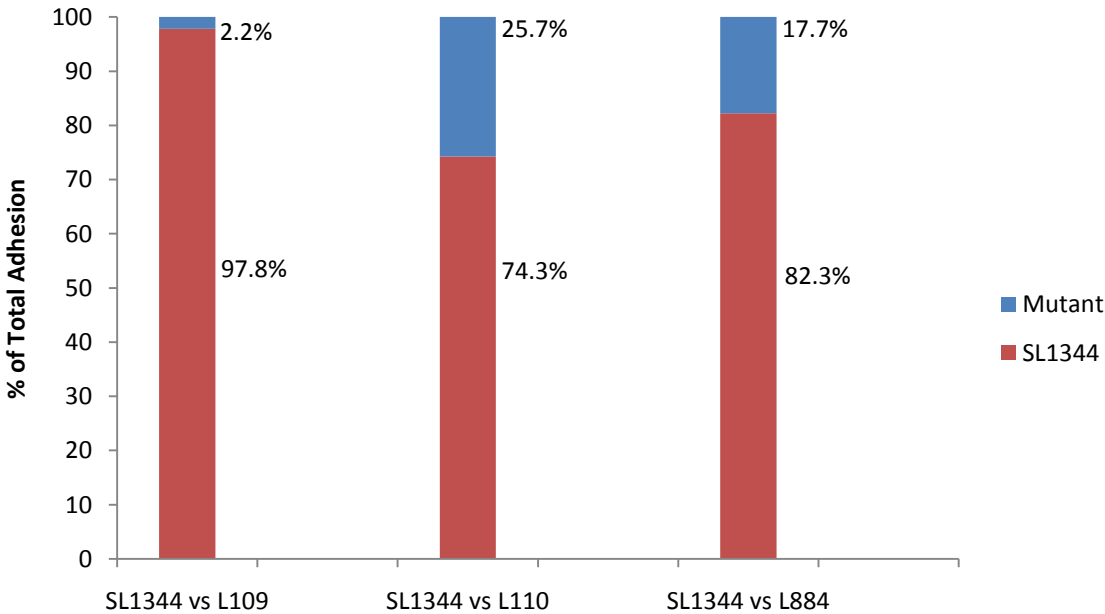
Following infection of INT-407 cells with a 50:50 mixture of SL1344 and the *acrB* mutant (L110) there was no significant difference between the mean number of each strain which adhered to the eukaryotic cells ($P = 0.29$) (Figure 5.7 a). There was also no significant

Figure 5.7 Adhesion of SL1344 in competition with L109 (*tolC::aph*), L110 (*acrB::aph*) or L884 (*acrA::aph*)

a. Adhesion values in CFU/ml for SL1344, L109 (*tolC::aph*), L110 (*acrB::aph*) and (*acrA::aph*) alone and the respective values of each when in competition.



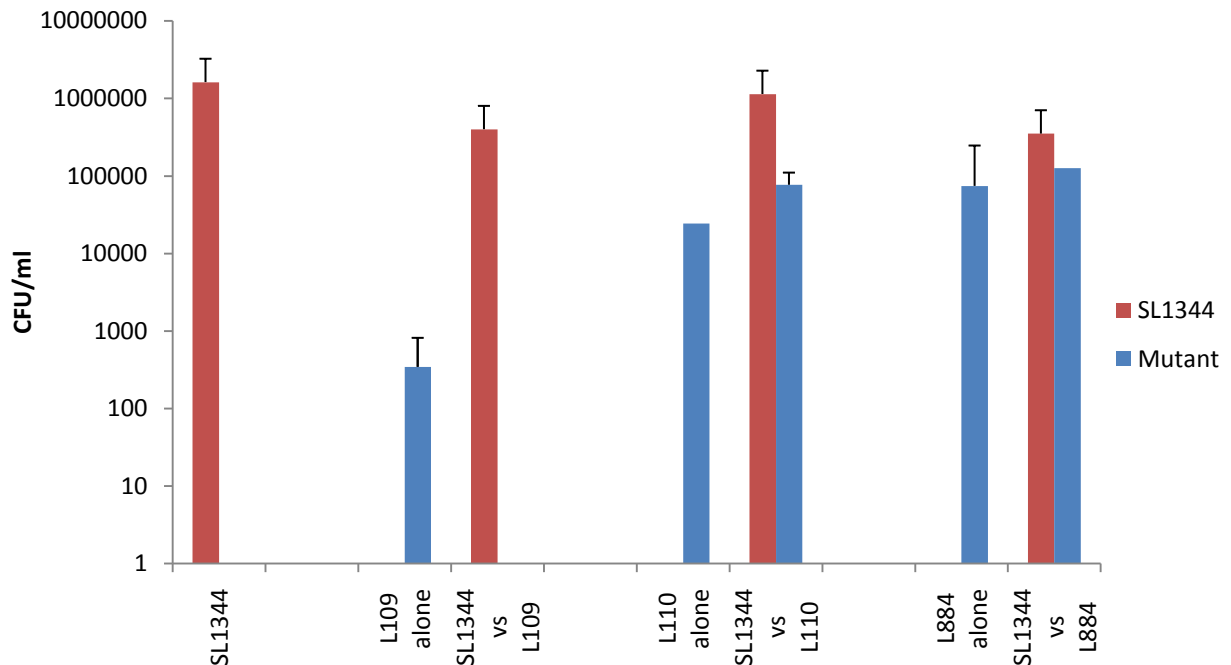
b. The percentage of the total adhesion for SL1344 and the respective mutant in each competitive infection



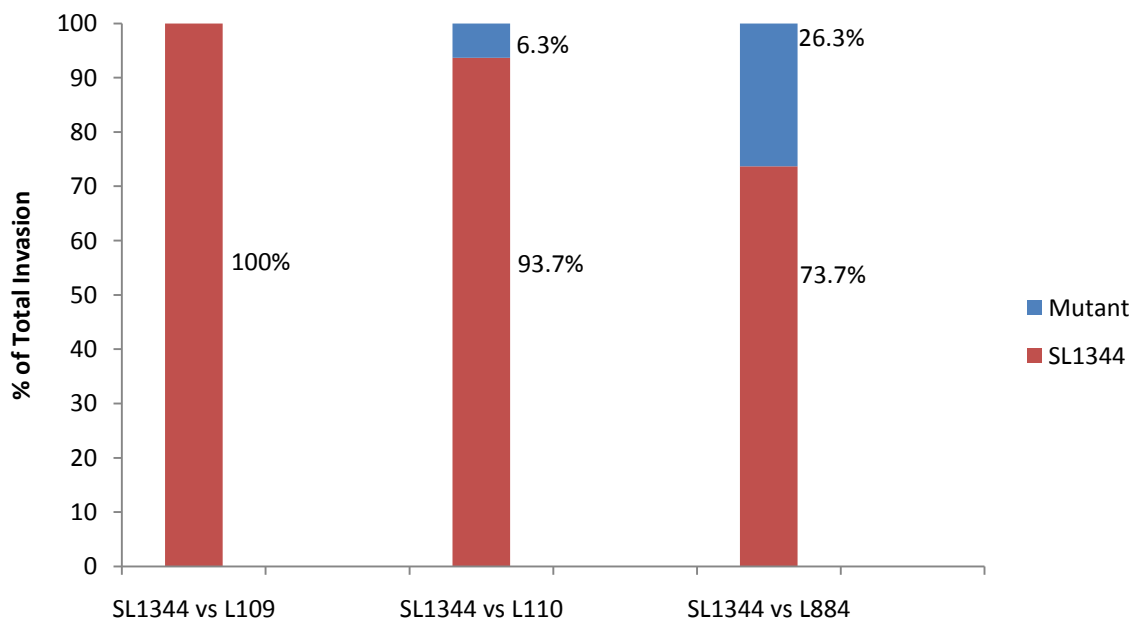
Values are expressed as mean of three experiments (performed in triplicate) + standard deviation.

Figure 5.8 Invasion of SL1344 in competition with L109 (*tolC::aph*), L110 (*acrB::aph*) or L884 (*acrA::aph*)

- a. Invasion values in CFU/ml for SL1344, L109 (*tolC::aph*), L110 (*acrB::aph*) and (*acrA::aph*) alone and the respective values of each when in competition.



- b. The percentage of the total invasion for SL1344 and the respective mutant in each competitive infection.



Values are expressed as mean of three experiments (performed in triplicate) + standard deviation.

difference between the mean number of L110 cells that adhered when L110 infected alone compared with the number which adhered during a mixed infection ($P = 0.30$) (Figure 5.7 a).

When a 50:50 mixture of SL1344 and L110 (*acrB::aph*) were used to infect INT-407 cells, significantly more SL1344 were able to invade ($P = 0.063$) (Figure 5.8 a) with the *acrB* mutant only accounting for 6.3% of the total invaded bacteria (Figure 5.8 b). However, the number of invaded L110 after mixed infection was not significantly different from the invasion level when L110 was inoculated alone. These data indicate that the invasion level of the mutant was not affected by the presence of the wild-type parental strain, SL1344. The number of SL1344 which adhered to or invaded the INT-407 cells was not significantly different when inoculated alone or in combination with the *acrB* mutant ($P = 0.23$ and $P = 0.28$, respectively) (Figure 5.7 and 5.8).

5.4.3 SL1344 and L109 (*tolC::aph*)

Following infection of INT-407 cells with a 50:50 mixture of SL1344 and L109 (*tolC::aph*), significantly fewer L109 adhered to the surface of the eukaryotic cells than did SL1344 ($p = 0.04$) (Figure 5.7 a). The mutant comprised only 2.2% of the total adherent bacteria (Figure 5.7 b). The numbers of adherent L109 (*tolC::aph*) were significantly less when INT-407 cells were co-infected with SL1344 than when they were infected with L109 (*tolC::aph*) alone ($P = 0.02$) (Figure 5.7 a).

When INT-407 cells were co-infected with SL1344 and L109 (*tolC::aph*), the *tolC* mutant was unable to invade as 100% of the invaded bacteria from the mixed infection were SL1344 ($P = 0.00003$) (Figure 5.8 a and b). These data for L109 are significantly different to those

obtained when L109 was inoculated alone ($P = 0.009$) (Figure 5.8 a). The numbers of SL1344 which adhered to, or invaded, the INT-407 cells were not significantly different when inoculated alone or with the *tolC* mutant ($P = 0.23$ and $P = 0.36$, respectively) (Figure 5.7 and 5.8).

5.5 Is AcrAB-TolC involved in efflux of a host derived factor which affects adhesion or invasion?

One hypothesis for the involvement of AcrAB-TolC in virulence and virulence related phenotypes is that it is involved in the transport of, or resistance to, a eukaryotic cell product and that inactivation or lack of components of AcrAB-TolC prevents this transport explaining the impaired virulence of the mutants. Giron and colleagues (2002) reported that lack of *perA* (a transcriptional activator), *luxS* (a quorum sensing autoinducer), *bfpA* (a type IV pilin) and type III secretion genes (including *espA*, *espB* and *espD*) had reduced production of flagella and subsequent reduced motility. The motility and flagella production of these *E. coli* mutants could be restored by growth in tissue culture medium conditioned by growth of HeLa cells. Therefore, the authors proposed that a eukaryotic cell signal had induced expression of flagellar related genes, which ameliorated the mutant phenotype (Giron *et al.*, 2002). To test the hypothesis that AcrAB-TolC is involved in the transport of, or resistance to, a eukaryotic cell product a similar experiment was performed. Inoculation media (MEM with 5% glutamine and 5% NEAA) was conditioned by incubation with uninfected INT-407 cell monolayers and then this media was used to dilute the bacterial suspensions to be used in infection assays.

To ensure that any exported factor was able to reach a sufficiently high concentration the time that fresh media was incubated with the INT-407 cells was explored. Fresh media was incubated with INT-407 cells for 30 minutes, 2 hours or 18 hours (overnight) in separate experiments, each performed in triplicate, to ensure sufficient time for any exported factor to reach a high concentration. The addition of INT-407 conditioned media harvested after 30 minutes, two hours or 18 hours incubation time had no significant effect on the ability of SL1344 or L109 (*tolC::aph*) to adhere to, or invade, INT-407 cells compared to the control infection in which bacteria were inoculated in basic inoculation media (Figure 5.9).

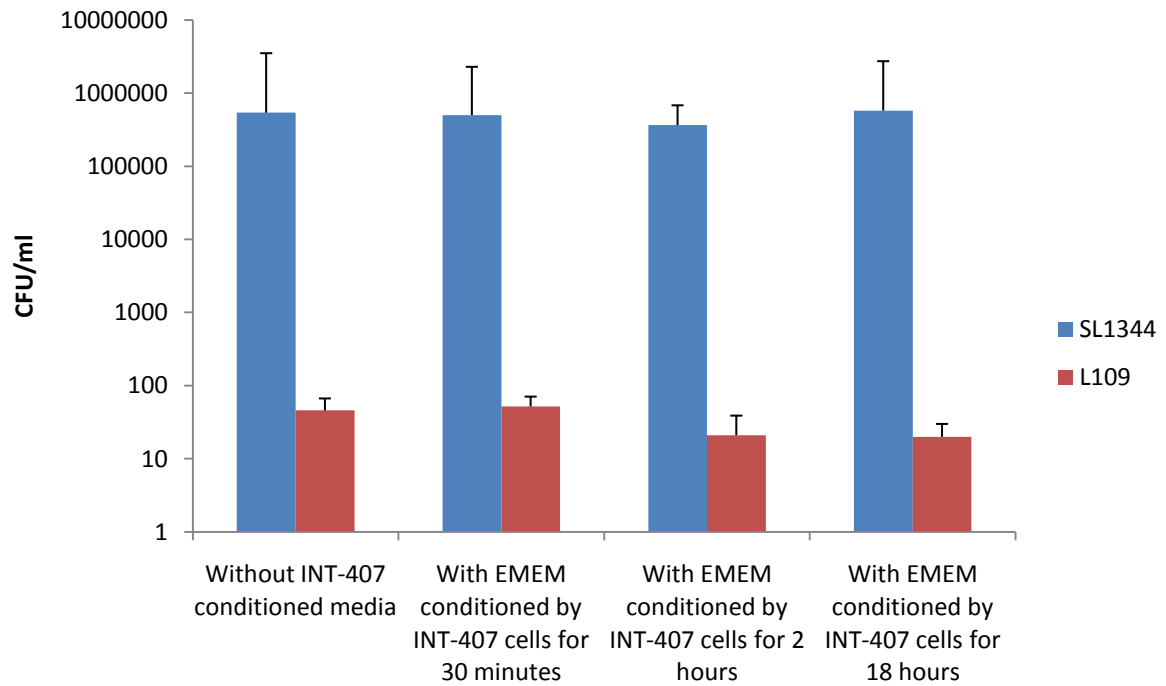
5.6 Is AcrAB-TolC involved in the efflux of a bacterially derived product or products which affect invasion? (Figure 5.10 - 5.13)

Another hypothesis for the involvement of AcrAB-TolC in virulence is that it is involved in the transport of a virulence related factor of bacterial origin. In this case it is possible that SL1344 is able to export this product, while the mutants either cannot export it or export it less efficiently, resulting in the invasion defect observed.

5.6.1 Can the invasiveness of the TolC mutant be restored by addition of media conditioned by growth of SL1344?

To investigate this hypothesis wild-type *Salmonella* (SL1344) or the *tolC* mutant were grown in LB medium overnight and, after filter sterilisation, this media was mixed 1:2 in inoculation media and used to dilute the wild-type or mutant bacterial suspension for standard infection assays as described in section 2.10.1. The premise of this experiment was that if SL1344 produced or exported a virulence related factor which was not produced or exported as

Figure 5.9 The effect of tissue culture media conditioned by INT-407 cells on the ability of SL1344 and L109 (*tolC::aph*) to invade INT-407 cells.

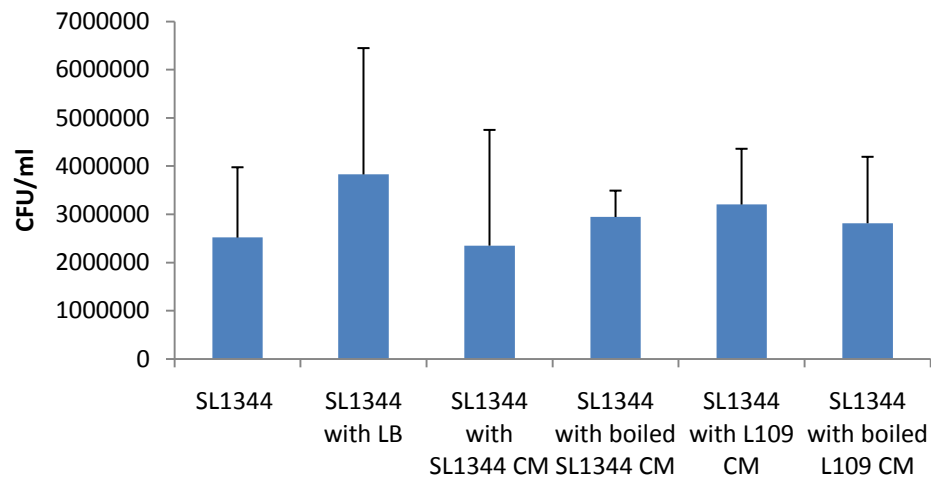


EMEM inoculation media was incubated with INT-407 cells for 30 minutes, 2 hours or 18 hours and filter sterilised using a low protein binding filter (0.2µm Millex®HV Durapore® PVDF membrane). The INT-407 conditioned media was then diluted 1:2 in inoculation media and used to dilute the bacterial suspension for the infection assay.

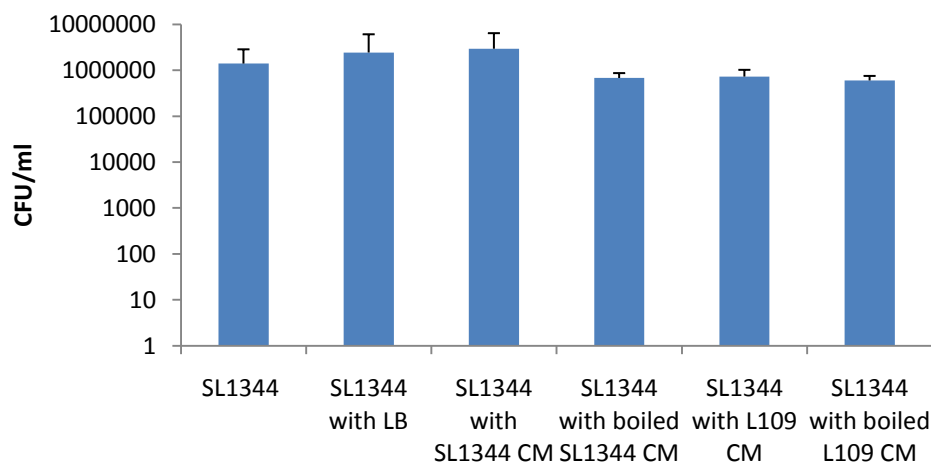
Data is displayed as mean of three separate experiments each performed in triplicate + standard deviation

Figure 5.10 The effect of bacterial culture media conditioned by growth of SL1344 or L109 (*tolC::aph*) on the ability of SL1344 to adhere to and invade INT-407 cells.

a. The effect of bacterially conditioned media on the adhesion of SL1344 to INT-407 cells.



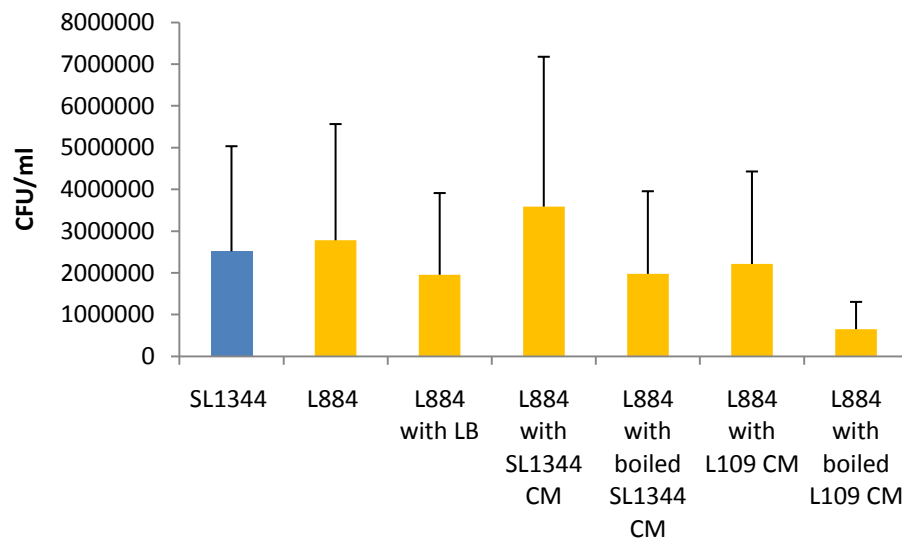
b. The effect of bacterially conditioned media on the invasion of SL1344 into INT-407 cells



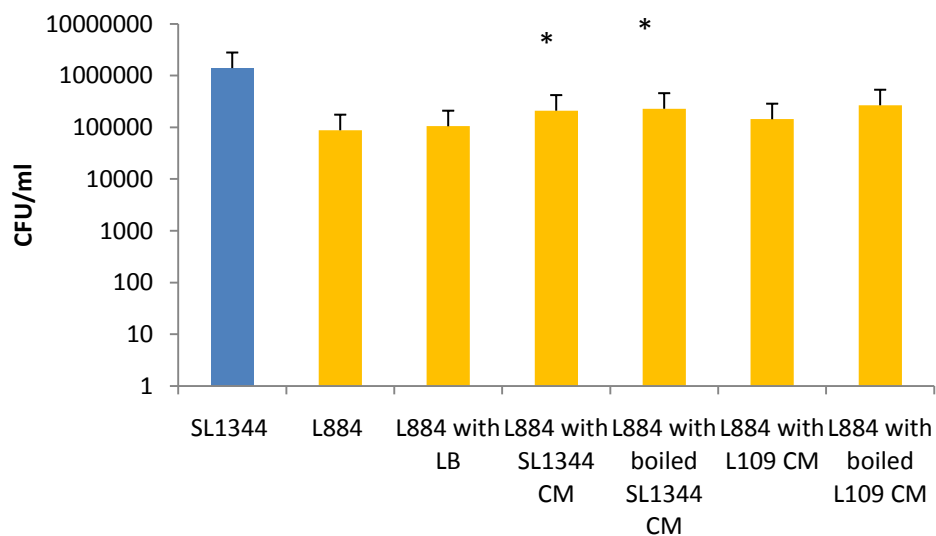
Data are displayed as mean of at least four separate experiments each performed in triplicate +/- standard deviation. CM = conditioned media.

Figure 5.11 The effect of bacterial culture media conditioned by growth of SL1344 or L109 (*tolC::aph*) on the ability of L884 (*acrA::aph*) to adhere to and invade INT-407 cells.

c. The effect of bacterially conditioned media on the adhesion of L884 (*acrA::aph*) to INT-407 cells.



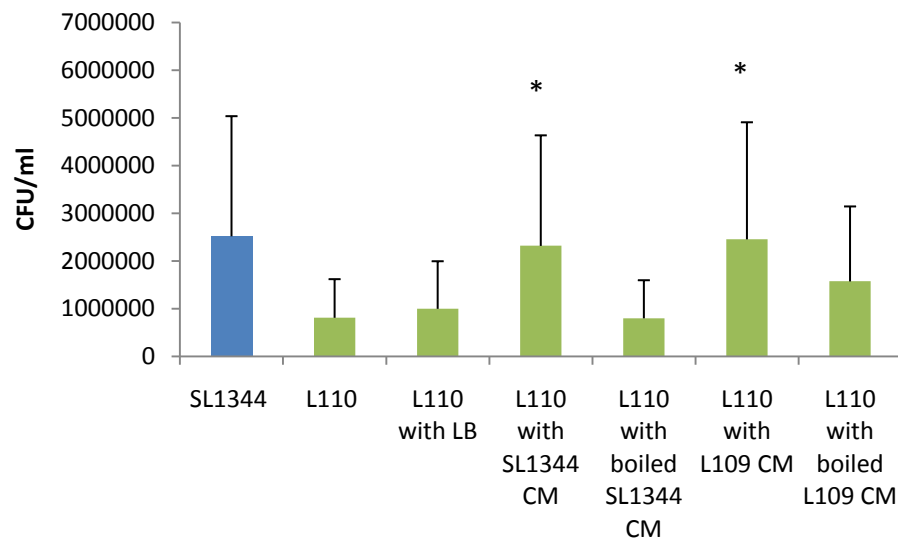
d. The effect of bacterially conditioned media on the adhesion of L884 (*acrA::aph*) into INT-407 cells.



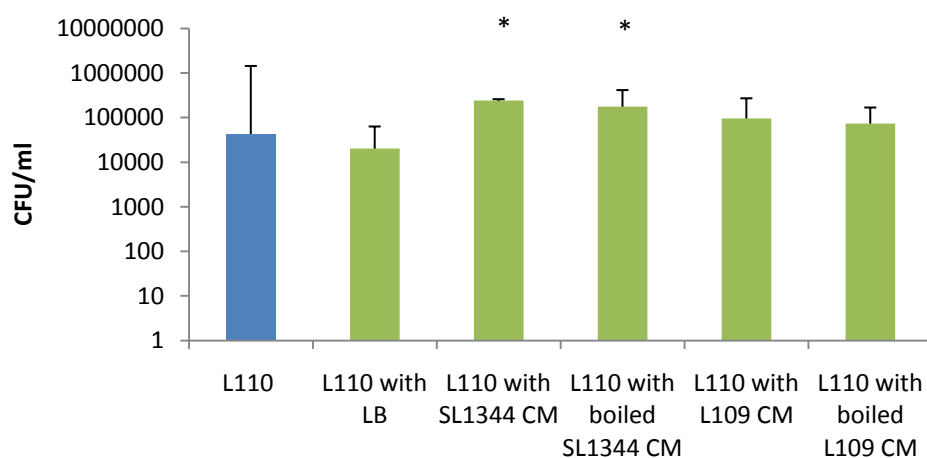
Data are displayed as mean of at least four separate experiments each performed in triplicate +/- standard deviation. CM = conditioned media.

Figure 5.12 The effect of bacterial culture media conditioned by growth of SL1344 or L109 (*tolC::aph*) on the ability of L110 (*acrB::aph*) to adhere to and invade INT-407 cells.

e. The effect of bacterially conditioned media on the adhesion of L110 (*acrB::aph*) to INT-407 cells.



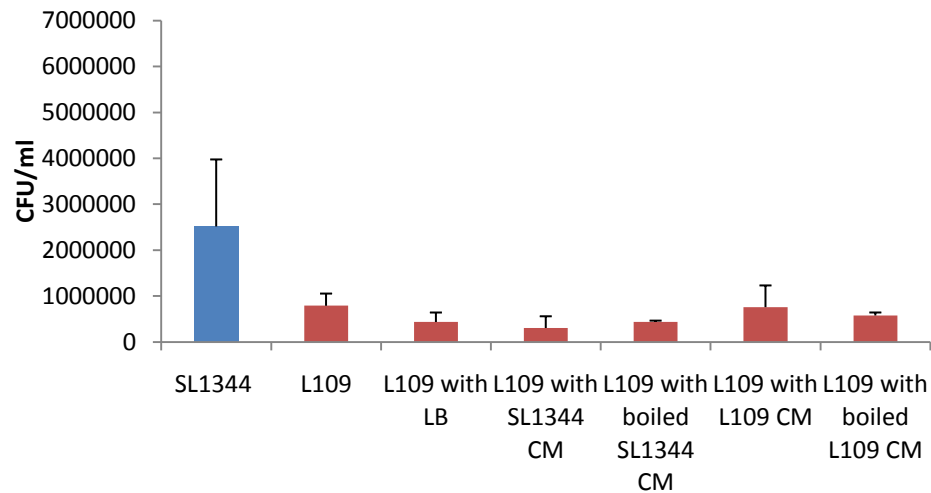
f. The effect of bacterially conditioned media on the invasion of L110 (*acrB::aph*) into INT-407 cells.



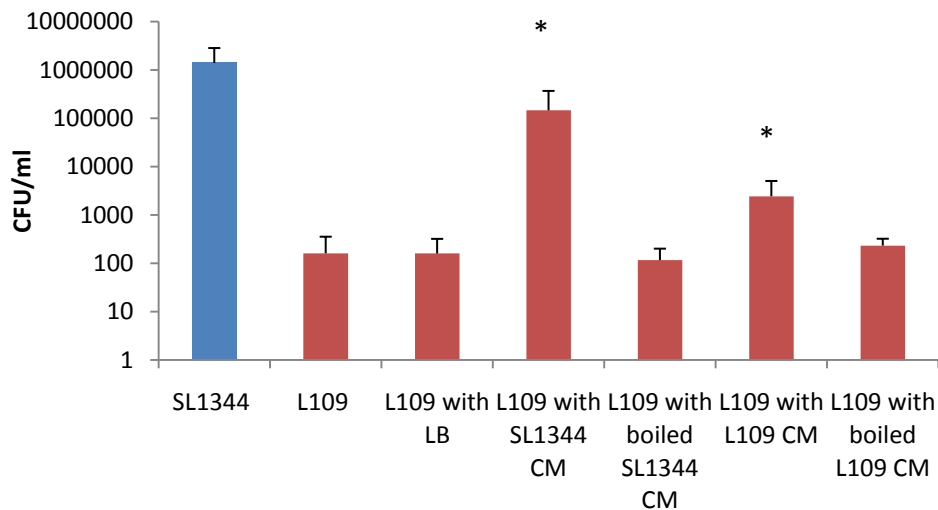
Data are displayed as mean of at least four separate experiments each performed in triplicate +/- standard deviation. CM = conditioned media.

Figure 5.13 The effect of bacterial culture media conditioned by growth of SL1344 or L109 (*tolC::aph*) on the ability of L109 (*tolC::aph*) to adhere to and invade INT-407 cells.

g. The effect of bacterially conditioned media on the adhesion of L109 (*tolC::aph*) to INT-407 cells.



h. The effect of bacterially conditioned media on the invasion of L109 (*tolC::aph*) into INT-407 cells.



Data are displayed as mean of at least four separate experiments each performed in triplicate +/- standard deviation. CM = conditioned media.

efficiently by the *tolC* mutant (L109) then addition of LB media conditioned by growth of SL1344 may be able to restore the invasion defect by exogenously providing the missing factor. Standard adhesion and invasion assays, as described previously (section 2.8.3 and 2.8.4), with the bacteria diluted only in fresh MEM inoculation media were performed in parallel. As a control the bacterial suspension was also diluted in a 1:2 mixture of fresh LB media with inoculation media to ensure that addition of LB broth alone did not affect the adhesion or invasion levels of SL1344 or the efflux mutants.

Levels of adhesion and invasion of SL1344, L109 (*tolC::aph*), L110 (*acrB::aph*) and L884 (*acrA::aph*) were not significantly different from the levels reported in chapter 4 (Figure 4.1).

The presence of LB in the inoculation media did not significantly alter the adhesion or invasion of SL1344 or any of the efflux mutants tested (L109, L110 and L884) (Figures 5.10 – 5.13).

The addition of media conditioned by the growth of either SL1344 or L109 (*tolC::aph*) had no significant effect on the levels of adhesion or invasion of SL1344 (Figure 5.10 a and b). The level of adhesion of the *acrA* mutant (L884) was also unaffected by the addition of media conditioned by either SL1344 or L109 (*tolC::aph*) (Figure 5.11 a). However, the mean level of invasion by L884 (*acrA::aph*) was significantly increased by addition of media conditioned by the growth of SL1344 by 2.39 fold ($P = 0.03$) but not by addition of media conditioned by the growth of the *tolC* mutant (L109) ($P = 0.17$) (Figure 5.11 b).

The addition of media conditioned by overnight growth of SL1344 was able to significantly increase adhesion and invasion of the *acrB* mutant (L110) to INT-407 cells 2.8 fold and 4.08

fold, respectively ($P = 0.05$ and 0.04 , respectively). Media conditioned by overnight growth of L109 also increased the level of adhesion of the *acrB* mutant 3.02 fold ($P = 0.01$). However, it did not affect invasion levels ($P = 0.21$) (Figure 5.12 a and b).

The level of adhesion of the *tolC* mutant (L109) was not significantly affected by the addition of media conditioned by either SL1344 or L109 (*tolC::aph*) (Figure 5.13 a). Addition of media conditioned by either SL1344 or L109 (*tolC::aph*) significantly increased the level of invasion by 905.56 fold and 15.11 fold, respectively ($P = 0.05$ and 0.01 , respectively) (Figure 5.13 b). However, the SL1344 conditioned media had a greater effect than the L109 conditioned media and the difference between the two was significantly different ($P = 0.0004$) (Figure 5.13 b).

5.6.2 Is the restorative effect of SL1344 conditioned media abolished by pre-boiling the conditioned media?

To determine whether the factor/s responsible for restoration of the virulence of the mutant strains was heat sensitive, LB media conditioned by growth of SL1344 and L109 was boiled for ten minutes and, after cooling, was used to dilute the bacteria for the infection assays in the same way as in section 2.10.1 to see if the effect was still evident. Infections in which SL1344, L109, L110 and L884 were inoculated in fresh MEM inoculation media and in SL1344 and L109 conditioned LB media were also performed in parallel.

Adhesion and invasion of strains inoculated in MEM inoculation media, SL1344 conditioned LB media or L109 conditioned LB media were not significantly different from the values already obtained. Boiled SL1344 conditioned LB media significantly enhanced the ability of

the *acrA* mutant (L884) to invade INT-407 cells ($P = 0.02$) (Figure 5.11 b) and there was no significant difference between the restorative effect of SL1344 conditioned LB media on the invasiveness of L884 before or after boiling ($P = 0.85$). The L109 conditioned LB media had no effect on the ability of the *acrA* mutant to adhere to or invade INT-407 cells and this was not significantly altered by boiling ($P = 0.07$) (Figure 5.11 a and b).

Boiling of the SL1344 conditioned LB media abolished its restorative effect on adhesion of the *acrB* mutant ($P = 0.94$). However, boiled SL1344 conditioned media was still able to increase the level of invasion of the *acrB* mutant (L110) ($P = 0.04$) and there was no significant difference between the restorative effect of SL1344 conditioned LB media on the invasiveness of L110 before or after boiling ($P = 0.66$) (Figure 5.12 a and b). The L109 conditioned media had no effect on the ability of the *acrB* mutant to adhere to, or invade, INT-407 cells and this was not altered by boiling the media ($P = 0.51$ and 0.16 , respectively) (Figure 5.12 a and b).

While both SL1344 and L109 conditioned LB media were able to, at least partially, restore the invasion defect of L109, boiling of either type of conditioned LB media abolished the restorative effect and resulted in invasion levels that were not significantly different from L109 invasion alone ($P = 0.67$ and 0.50 , respectively) (Figure 5.13 b).

5.6.3 Is the restorative effect of SL1344 conditioned media abolished by treatment with Proteinase K?

To determine whether the molecule or molecules responsible for restoration of the virulence of the mutant strains are proteinaceous, filter sterilised LB media conditioned by

growth of SL1344 and L109 were treated with proteinase K, a broad spectrum protease. The enzyme was then inactivated with ethylene glycol tetraacetic acid (EGTA) and after dilution 1:2 with inoculation media the proteinase K treated media was used to dilute the bacteria for the infection assays in the same way as in section 2.10.1. It was postulated that if the molecule or molecules responsible for the effect are proteinaceous, then the effect would be abolished by proteinase-K treatment. Infections in which SL1344, L109, L110 and L884 were inoculated in fresh tissue culture inoculation media and in SL1344 and L109 conditioned LB media were also performed in parallel.

Adhesion and invasion of strains inoculated in MEM inoculation media, SL1344 conditioned LB media or L109 conditioned LB media were not significantly different from the values already obtained. However, no adhesion or invasion was detected for SL1344 or the mutants (L109, L110 or L884) in experiments with addition of SL1344 or L109 conditioned LB media which had been treated with proteinase K. Data is not shown for these experiments as visible destruction of the monolayer during the experiment made counting of bacterial colonies and interpretation of data impossible.

5.6.4 The effect of the bacterially conditioned media on the number and viability of INT-407 cells.

To determine whether the bacterially conditioned media affected the number and viability of INT-407 cells in the monolayers, the monolayers were incubated with SL1344 and L109 conditioned LB media, boiled SL1344 and L109 conditioned LB media or Proteinase K treated SL1344 and L109 conditioned LB media for two hours to simulate the conditions during an assay. No bacteria were present. The monolayers were all washed three times with HBSS

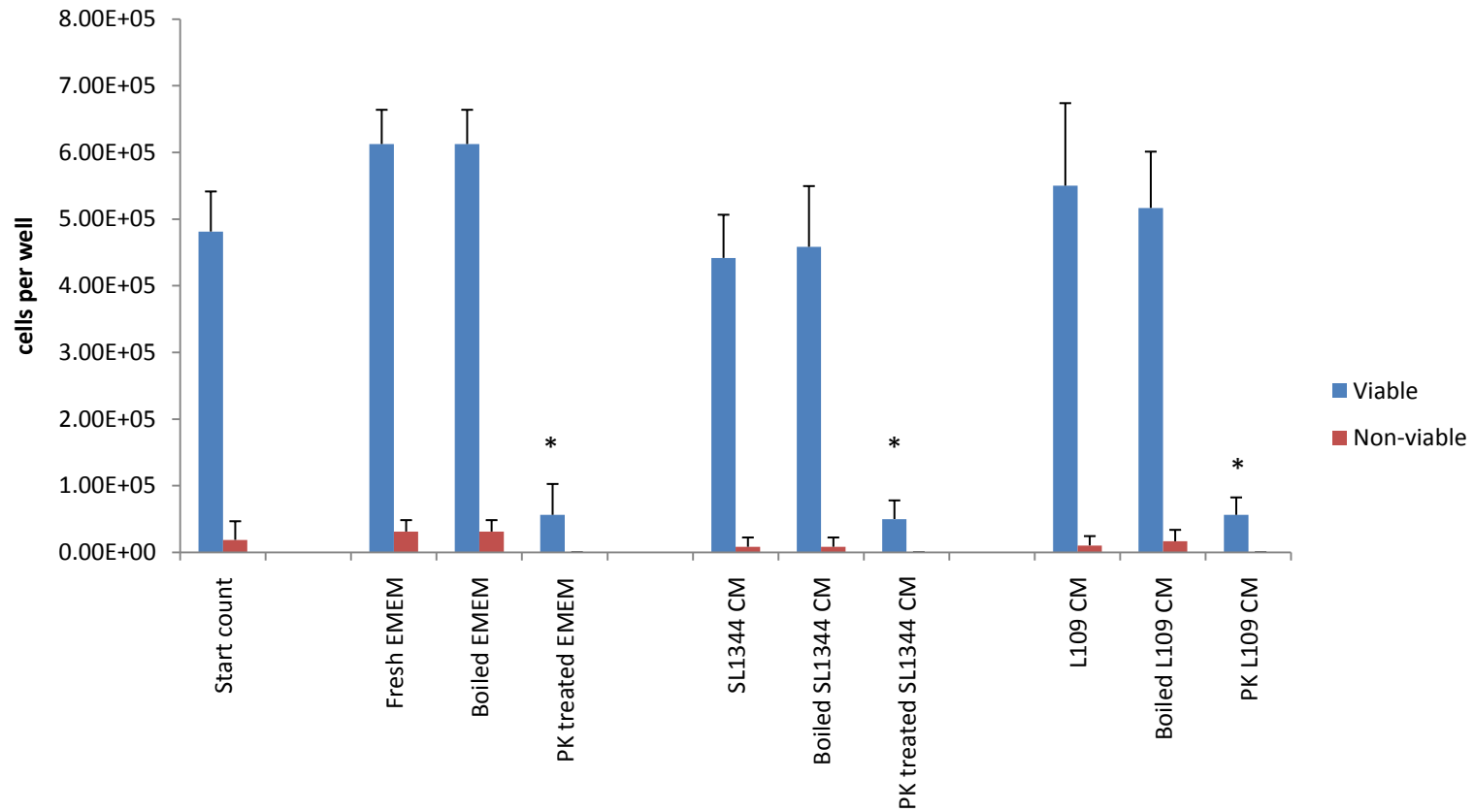
before and after the incubation to simulate the protocol used during the assays. Viability of the cells was tested by addition of trypan blue before counting.

The number of cells at the start of the experiment was within the normal range ($4-6 \times 10^5$ cells per well) and as used in previously published experiments (Dibb-Fuller *et al.*, 1999). Two hours of incubation with fresh MEM inoculation media (with 5% Glutamine, 5% NEAA) or media conditioned by the growth of either SL1344 or L109 had no significant effect on the number of INT-407 cells present ($P = 0.06, 0.24$ and 0.62 , respectively) or the number of non-viable cells present in each well ($P = 0.68, 0.39$ and 0.39 , respectively) (Figure 5.14). Incubation for two hours with MEM, SL1344 conditioned media or L109 conditioned media that had been boiled for 10 minutes also had no significant effect on the number ($P = 0.07, 0.46$ and 0.89 , respectively) or viability ($p = 0.70, 0.38$ and 0.47 , respectively) of INT-407 cells (Figure 5.14). However, incubation for two hours with MEM, SL1344 conditioned media or L109 conditioned media that had been pre-treated with proteinase K which was then been inactivated with EGTA, significantly reduced the number of cells per well ($P = 0.00004, 0.00002$ and 0.0002 , respectively) although cell viability was not significantly affected ($P = 0.17, 0.27$ and 0.17 , respectively) (Figure 5.14).

5.7 Discussion

In addition to an established role in antimicrobial resistance, MDR efflux pumps, including AcrAB-TolC, have been shown contribute to the basic biology of various pathogens (Piddock, 2006). RND systems are required for the virulence of several species including *S. enterica* and *P. aeruginosa* as mutants lacking functional RND efflux systems are attenuated (Hirakata *et al.*, 2002; Buckley *et al.*, 2006; Nishino *et al.*, 2006; This study).

Figure 5.14. The effect of bacterially conditioned LB medium, boiled bacterially conditioned LB medium and Proteinase-K treated bacterially conditioned LB medium on the number and viability of INT-407 cells per well.



Viable and non-viable cells were differentiated using trypan blue. Data are displayed as mean of three separate experiments each performed in triplicate + standard deviation. PK, Proteinase K treated. CM, conditioned medium

A recent study from this laboratory showed that one explanation for the attenuation of RND efflux mutants is changes in expression of virulence associated genes and concomitant changes in effector protein expression, motility and anaerobic metabolism. For example strains lacking AcrB or TolC had reduced expression of genes found on SPI-1 and subsequently reduced production of the effector proteins SipA, SipB and SipC. The *acrA* mutant was distinct from strains lacking AcrB and TolC in that SPI-1 expression was unchanged but repression of genes found in SPI-2 was evident (Webber *et al.*, 2009). Other changes included reduced expression of genes involved in chemotaxis and motility after inactivation of *acrB* or *tolC*, and in the *acrB* mutant this was associated with diminished motility. Furthermore, after inactivation of *acrA* or *acrB* repression of the *nap* and *nir* operons were seen which adversely affected growth in anaerobic conditions compared to the parental strain, SL1344 (Webber *et al.*, 2009).

An alternative explanation for the involvement of RND systems in pathogenicity came from work with *P. aeruginosa* which showed that MexAB-OprM is responsible for export of a factor required for bacterial virulence. However, the two hypotheses are not mutually exclusive.

Contrary to the findings of Hirakata and colleagues with *P. aeruginosa*, the addition of supernatant from tissue culture cells infected with SL1344 had no effect on the ability of the *tolC* mutant (L109) to adhere to, or invade, INT-407 cells *in vitro* (Figure 5.2 - 5.6). This suggests that the involvement of AcrAB-TolC in pathogenicity may not be mediated by the efflux of an exported virulence related factor, as was suggested of *P. aeruginosa*. However, the possibility remains that either it is not possible to detect rescue of invasive ability in the

same way in *Salmonella* or that some detail of the experiment prevented detection of the rescue in the assays performed. For example, Hirakata and colleagues used a triple MexAB-OprM mutant while a triple *acrAB-tolC* SL1344 mutant was not available at this time. This may have made detection more difficult. It is also possible that any virulence related factor was not able to reach a sufficiently high concentration during this experiment to effect virulence.

The hypothesis that AcrAB-TolC exports a virulence related factor, which is not exported by the mutant, was also investigated by infecting INT-407 cells with a 50:50 mixture of SL1344 and one of the efflux pump mutants. The aim was to determine whether co-infection with SL1344 was able to complement the fitness defect of the mutant by supplying an exported virulence related factor which the mutant was not able to export. Previously, competitive index experiments in poultry showed that when chickens were infected with a 1:1 mixture of SL1344 with either an *acrB* mutant or a *tolC* mutant that the mutants were out-competed by SL1344 (Buckley *et al.*, 2006). Mutants lacking AcrB were able to colonise the avian gastrointestinal tract but were unable to persist while lack of TolC rendered the bacteria unable to colonise or persist. This is in accordance with the findings of the adhesion and invasion tissue culture experiments (Figure 4.1) showing that the *acrB* mutant (L110) was able to adhere but not invade as well as SL1344 and that the *tolC* mutant (L109) had a reduced ability to both adhere to and invade intestinal cells compared to SL1344. These experiments were replicated in the *in vitro* tissue culture model for all three mutants.

After a mixed infection of SL1344 and the *acrA* mutant (L884), the mutant accounted for significantly less of the total invaded bacteria. The level of invasion by L884 was not

significantly different from when L884 alone was used to infect INT-407 cells so the invasion defect of L884 had not been complemented by the presence of SL1344. It did not represent out competition by SL1344 because the level of L884 invasion reflected the level of attenuation seen in a single infection. Similarly, when INT-407 cells were infected with a mixture of SL1344 and the *acrB* mutant (L110), the level of invasion was not significantly different from when L110 alone was used to infect INT-407 cells so the invasion defect of the *acrB* mutant had not been complemented by presence of the wild-type. Again, the mutant strain was not out-competed by SL1344 as the levels of attenuation seen reflected levels when the *acrB* mutant infected alone. Only the *tolC* mutant (L109) was truly out-competed when inoculated in a mixed infection with SL1344. Not only was there a significantly lower mean number of adhered and invaded bacteria, but the levels were significantly lower than when L109 was used to inoculate INT-407 cells alone. This is congruent with earlier *in vivo* data where the *tolC* mutant was shown to be out-competed by SL1344 in the avian gastrointestinal tract (Buckley *et al.*, 2006). However, in the tissue culture experiments these data cannot be explained by the inability of L109 to withstand bile concentrations.

The attenuation seen in the efflux mutants could not be complemented by either addition of supernatant from SL1344 infected INT-407 cells or co-infection with SL1344. These data suggest that there is no virulence related factor exported by wild-type, that is lacking in the mutant, which is able to restore the fitness defect of the mutant when supplied exogenously.

In contrast, Hirakata and colleagues concluded from their results that MexAB-OprM (an orthologue of AcrAB-TolC) exports “virulence determinants that contribute to bacterial

virulence.” However, there are several plausible alternative interpretations of this data, two of which have been investigated during this study. The first is that the factor affecting invasion originates from the eukaryotic host cells and that the mutants cannot tolerate or respond in the same manner as the wild-type, leading to lower infection levels. This hypothesis fits with *in vitro* evidence showing that the *E. coli* AcrAB-TolC system is involved in transport of, and resistance to, several host derived substrates including bile, fatty acids and steroid hormones (Ma *et al.*, 1995; Thanassi *et al.*, 1997; Elkins and Mullis, 2006; Drew *et al.*, 2008). A similar phenomenon was reported by Giron and colleagues (Giron *et al.*, 2002) who found that the reduced motility and flagella production of *E. coli* mutants lacking PerA (a transcriptional activator), LuxS (a quorum sensing autoinducer), BfpA (a type IV pilin) and certain T3SS proteins (including EspA, EspB and EspD) was restored after growth in tissue culture medium conditioned by growth of HeLa cells. This study proposed that a eukaryotic signal had induced gene expression changes have affected mutant phenotypes. However, evidence presented herein suggests that an exported host cell signal is not the reason that the *tolC* mutant is attenuated as addition of INT-407 conditioned media did not restore the ability of the *tolC* mutant to invade (Figure 5.3).

The second alternative interpretation of their findings is that the ‘virulence determinant’ is a normal product of the wild-type bacteria, not exclusively produced during infection, but which is not produced, or not efficiently exported, by the efflux mutants. In this scenario the product could be proteinaceous or possibly a metabolic product which either directly effects invasion or build up of which is toxic or inhibitory. The restoration of the attenuated phenotype of the efflux mutants by SL1344 conditioned media supports the hypothesis that a compound produced by SL1344, but not the *tolC* mutant, was required for invasion.

As addition of LB media conditioned by wild-type bacteria (SL1344) was able to restore the invasive defect of the mutants, it is somewhat surprising that the same restoration was not seen by addition of the supernatant from INT-407 cells infected with SL1344 or in the competition assays in which the INT-407 cells were co-infected with one of the mutants and SL1344. It is possible that the effective factor did not reach a sufficiently high concentration in these experiments or the competition experiments for the effect to be detected. This may simply be because the number of bacterial cells per millilitre present in an overnight culture is much higher than in an infection experiment and the bacteria are grown for much longer to produce the bacterially conditioned media than the time they are present in the tissue culture experiments. Another explanation is that the supernatant from infected INT-407 cells contains “cell death” signals from infected eukaryotic cells. These could mask the effect of any virulence factor. Conversely, the effect of bacterially conditioned media was not seen by Hirakata and colleagues when LB broth was conditioned by the growth of either the wild-type or mutant *P. aeruginosa*. The discrepancy between these two sets of results is noteworthy but may be at least partially explained by the biological differences between *S. enterica* and *P. aeruginosa*.

The virulence factor or factors may be proteinaceous. However, pre-boiling the SL1344 conditioned media did not ablate its restorative effect on L884 (*acrA::aph*) and L110 (*acrB::aph*) suggesting that the product is unlikely to be proteinaceous. However, the boiled SL1344 conditioned media was unable to complement the invasion defect in L109 (*tolC::aph*). Unfortunately, it was not possible to confirm whether the factor was proteinaceous due to the failure to successfully treat the conditioned media with proteinase K at this time. It is likely that the problem with this digestion lies in the inactivation step.

Heat could not be used to inactivate the proteinase as earlier results had shown that boiling the media altered its ability to complement the invasion defect in L109 (*tolC::aph*). There are three compounds suggested by the manufacturer (Promega) for inactivation of Proteinase K; phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP) or ethylene glycol tetraacetic acid (EGTA). Of which, PMSF was unsuitable as it is highly cytotoxic and therefore likely to damage or kill the eukaryotic cells. DFP is a potent neurotoxin so, along with PMSF, could not be used for safety reasons as both compounds must be used only in the fume cupboard making the incubation steps required in the tissue culture experiments impossible. EGTA was deemed safe to use in these assays and thought unlikely to affect the eukaryotic cells as it has been used in numerous *in vitro* studies with eukaryotic cells and *in vivo* animal tests (Wang *et al.*, 2000). Proteinase K is stabilised by calcium and is inhibited by EGTA because it chelates calcium. However, the manufacturer's guidelines state that after inhibition by removal of calcium up to 20% of the catalytic activity of the proteinase K could remain. This incomplete inactivation probably led to destruction of the monolayer. An alternative proteinase, which can be completely inactivated without heat, will need to be used to determine whether the factor is proteinaceous.

While pre-boiling the SL1344 conditioned LB media ablated its restorative effect on L109 (*tolC::aph*) invasion, the ability to restore invasion of L884 and L110 was not affected. This suggests that it may be a different factor or combination of factors which is restoring the invasive ability of L109. It is possible that additional factors are required to restore the invasive ability of the *tolC* mutant as TolC functions with numerous other protein partners, and the attenuation of one of these functions may contribute to the loss of invasive ability.

In that case an additional factor could be involved in the restoration seen after addition of SL1344 conditioned media and this factor could be modified by boiling.

Previously, media pre-conditioned by bacterial growth has been used to study quorum sensing (Sperandio *et al.*, 2001). Quorum sensing signals (QSS) are molecules produced by bacteria whose concentration increases with increasing bacterial cell density effectively allowing cell-cell communication (Miller and Bassler, 2001). In Gram-negative bacteria QSSs are normally acylated homoserine lactones. These molecules act as autoinducers which, above a threshold concentration, affect the expression of genes controlling numerous bacterial processes including biofilm formation, competence, conjugation and virulence (Miller and Bassler, 2001; Waters and Bassler, 2005). In the case of quorum sensing studies, media pre-conditioned by the growth of bacteria is used to provide the QSS itself. Yang *et al.*, postulated that *E. coli* AcrAB-TolC effluxes quorum sensing molecules (Yang *et al.*, 2006). It was shown that *E. coli* lacking AcrAB (LZ2184) had a similar growth rate to the wild-type strain but grew to a higher final optical density in stationary phase. It was shown that media conditioned by growth of the wild-type and to a greater extent by an *acrAB* over-expressing strain was able to inhibit growth of *E. coli* but that media pre-conditioned by growth of the *acrAB* mutant could not. From this, the authors concluded that QSSs are natural substrates of AcrAB-TolC. However, there is no evidence that this is the case for *Salmonella* as no over-growth seen in *Salmonella* Typhimurium strains lacking AcrA, AcrB or TolC (Bailey *et al.*, 2008, Webber *et al.*, 2009, This study). It was thought this may be explained by differences between the quorum sensing systems of *E. coli* and *S. Typhimurium* (Ahmer *et al.*, 1998). However the experiment was repeated using the *E. coli* strains W4573 (WT) and LZ2184

(Δ *acrAB*) which were used in the study by Yang *et al.*, (2006). The overgrowth effect could not be reproduced in our lab using the strains used in the published work so it was concluded that it is unlikely that AcrAB-TolC is involved in the transport of QSS and therefore that QSSs do not account for the restoration of L109 (*tolC::aph*) virulence by SL1344 conditioned media (Webber *et al.*, 2009, supplementary information).

It is possible that AcrAB-TolC could export a virulence related factor which is a metabolite or other small soluble factor. It has been suggested that TolC of *E. coli* O42 exports such a factor. The O42*tolC* strain did not aggregate in the same way as the wild-type strain as more planktonic cells, and less precipitated cells, were present in a micro-titre plate assay. The aggregative phenotype of the wild-type is known to be important for virulence. The transwell system consisting of a 0.4 μ m-pore-size polyester filter insert for a 24 well tissue culture assay plate was used. This filter sits inside the well and allows co-culture of the wild-type, O42, and the mutant O42*tolC*, in the same well with secreted proteins and cytokines able to pass through the polyester membrane, while bacteria are kept separate. When the mutant, O42*tolC* was co-cultured with the wild-type, more precipitated cells were present and less planktonic cells showing that the aggregation defect of the *tolC* mutant had been complemented by presence of the wild-type (Imuta *et al.*, 2008). The authors concluded that TolC must secrete a humoral factor able to complement this mutant phenotype which is important for virulence. Rosner and Martin recently hypothesised that in *E. coli* intracellular accumulation of toxic bacterial metabolites during growth led to up-regulation of MarA, SoxS and Rob, which stimulates up-regulation of *tolC* to remove the metabolites (Rosner and Martin, 2009).

Recently, Huang *et al.*, (2008) showed that a *Salmonella* strain deficient in two key metabolic genes, acetate kinase and phosphotransacetylase (*ackA-pta*), had reduced SPI-1 expression and was less able to invade Hep-2 cells. The invasive phenotype could be restored by addition of media conditioned by growth of the wild-type strain (Huang *et al.*, 2008). In this case the authors found that the mutant strain excreted 68-fold less formate than the parent and it was also possible to restore virulence and SPI-1 expression by addition of exogenous sodium formate. Furthermore, the mouse distal ileum, the region of the gastrointestinal tract into which *Salmonella* invades, was found to have the appropriate concentration of formate found to induce expression of genes involved in invasion *in vitro*, while the formate was undetectable in the cecum (Huang *et al.*, 2008). This study shows that the factor allowing SL1344 conditioned media to restore the ability of L109 to invade could be formate, or another small diffusible molecule or metabolite.

5.8 Further work

Firstly, to allow investigation of whether the molecule effecting virulence of the mutants is proteinaceous alternatives to proteinase K will be investigated.

The study of bacterially conditioned media will be extended to include the effect of media conditioned by the *acrA* mutant and the *acrB* mutant on the ability of each of the mutants to adhere to and invade INT-407 cells.

Further investigation of the hypothesis that AcrAB-TolC exports a factor required for virulence-related phenotypes has begun. To confirm export of a bacterially derived molecule, an *S. Typhimurium* mutant is being constructed in which the *tolC* gene is present

but contains two single nucleotide polymorphisms such that the protein produced will be locked closed and unable to efflux substrates. This will be based upon a paper by Eswaran and colleagues where a locked TolC was created in *E. coli* and used to investigate the mechanism of the efflux pump (Eswaran *et al.*, 2003). Once a locked TolC has been created in *Salmonella* the ability of the strain to adhere to and invade INT-407 cells will be determined and conditioned media assays will be performed.

Recently, Hirakata and colleagues (2009) found that PA β N and a MexB specific inhibitor, D13-9001, were able to reduce the invasiveness of *P. aeruginosa in vitro* (Hirakata *et al.*, 2009). Previous work in *Campylobacter* also showed that oral administration of PA β N to chickens 2-4 days post inoculation with *C. jejuni* significantly reduced colonisation as did inactivation of CmeABC, a homologue of AcrAB-TolC (Lin and Martinez, 2006). Association and invasion assays will be performed with SL1344 and the three AcrAB-TolC mutants in the presence of various concentrations of PA β N. It is thought that PA β N also inhibits AcrB, so these experiments will allow confirmation that the attenuation seen in the efflux mutants is due to loss of efflux function and not just as a result of other virulence related gene changes.

NMR and Mass spectrometry experiments on SL1344, L109 (*tolC::aph*) and L110 (*acrB::aph*) conditioned media are currently under way to identify any differences between the exported metabolites from the wild-type, *acrA*, *acrB* and *tolC* mutants. The aim is to identify any differences in the metabolites present and eventually to add these exogenously to the infection assays to see whether the virulence defect in the mutants can be restored. This study should also allow identification of some natural substrates of AcrB and TolC.

5.9 Key findings

- The levels of adhesion and invasion of a *tolC* mutant could not be restored by the addition of supernatant from an infection of INT-407 cells with SL1344 as has previously been shown for a *mexAB-oprM* mutant in *P. aeruginosa*.
- The invasion defect seen in mutants lacking AcrA, AcrB or TolC could not be complemented by co-infection with SL1344.
- When INT-407 cells were co-infected with SL1344 and the *tolC* mutant, the *tolC* mutant was outcompeted: adhesion levels were reduced compared to infection with L109 alone and invasion was abolished. While neither the *acrA* nor *acrB* mutant was out-competed when INT-407 cells were co-infected with SL1344 and either mutant.
- The levels of adhesion and invasion of a *tolC* mutant could not be restored by the addition of inoculation media conditioned by incubation with INT-407 cells.
- LB media conditioned by the growth of SL1344 was able to restore the invasive defect of the *acrA*, *acrB* and *tolC* mutant.
- Boiling the SL1344 conditioned media did not affect its ability to restore the invasive ability of the *acrA* or *acrB* mutants but abolished its restoration of the ability of the *tolC* mutant to invade INT-407 cells.

Chapter 6

Overall Discussion and Conclusions

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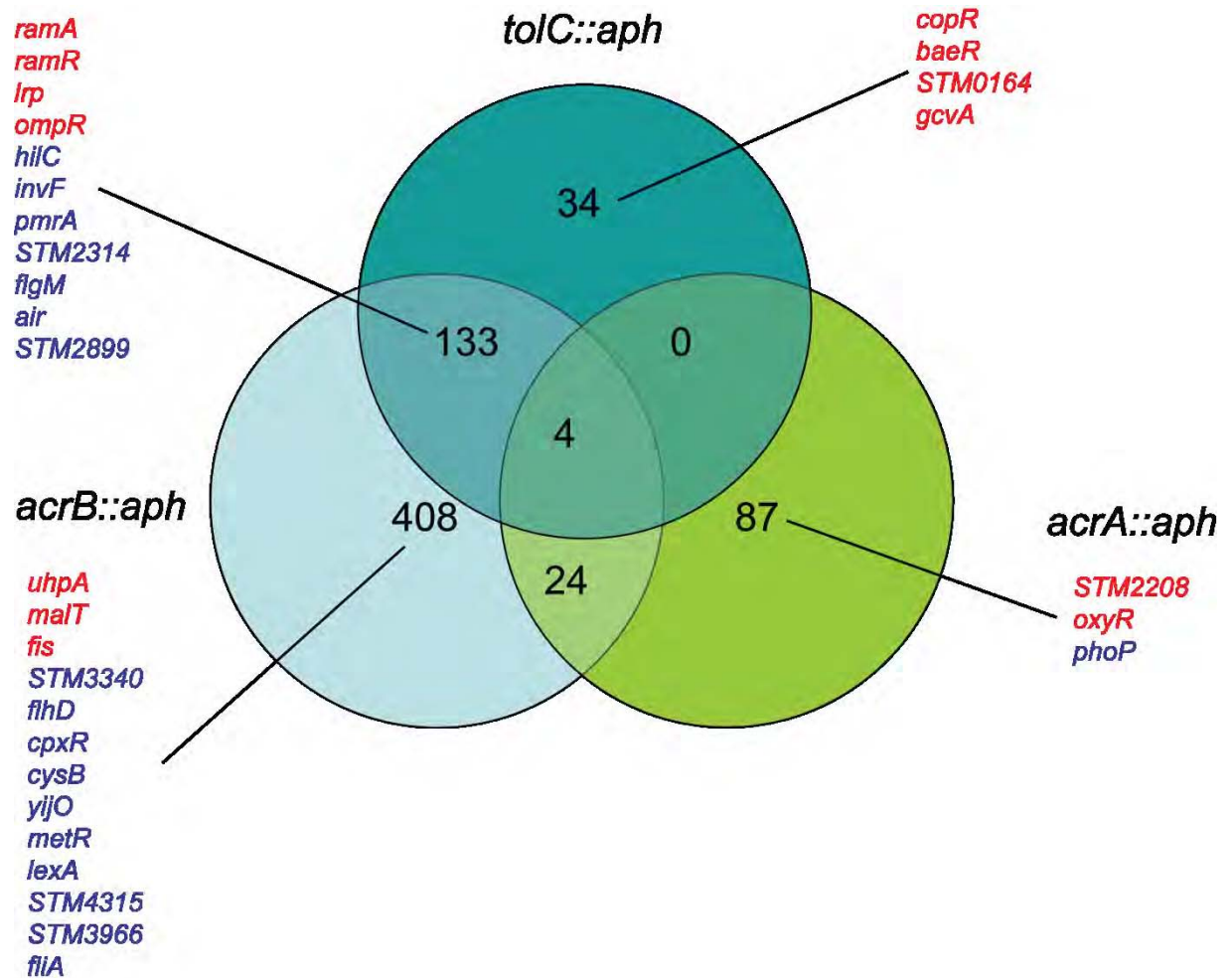
In this study the periplasmic adaptor protein AcrA of *Salmonella enterica* serovar Typhimurium was inactivated. The strain lacking this protein accumulated significantly more Hoechst H3342 than the wild-type strain SL1344 (Figure 3.10) showing that efflux activity in this strain was impaired. This is complementary to recent structural data based on extensive site-directed cross-linking studies showing that AcrA has an important structural role in the AcrAB-TolC efflux pump (Symmons *et al.*, 2009). The model suggests that the interaction between TolC and AcrA is most energetically favourable when the TolC channel is in its open state. Once AcrA is bound to AcrB, TolC is recruited to the complex causing TolC to open. The outer membrane channel then remains in a constitutively open state which allows the high throughput efflux which is characteristic of this system. In this model the closure of TolC is not required as the weak interaction between the AcrB and TolC trimers is stabilised by three AcrA molecules meaning that there is no leakage of substrates into the periplasm (Symmons *et al.*, 2009). Based on this model, it is postulated that lack of AcrA may prevent assembly of the trimer. Alternatively, AcrB and TolC may be able to interact, but this interaction may be weak and the open state of TolC may not be properly maintained. This could lead to significantly diminished efflux levels or even leakage of the substrates into the periplasm. It remains a possibility that lack of any of the components of the pump is partially complemented by homologues of the system. For example, it is possible that some transport through AcrB and TolC could be supported by the periplasmic adaptor protein AcrE which shares 66% identity and 80% similarity with AcrA.

The reduction in efflux level partially explains the increased susceptibility of the *acrA* mutant to a range of antimicrobials because if toxic compounds are not efficiently extruded they will accumulate inside the cell and are lethal at lower concentrations. However, the effect was substrate specific as the *acrA* mutant was the most susceptible to triclosan while it did not have the most attenuated efflux level as measured by accumulation of Hoechst H3342. It has been suggested that the PAP may

have a role in facilitating recruitment of hydrophobic substrates to the pump from the periplasm due to the proximity of the newly modeled membrane proximal domain to the binding pocket identified between the PN2 and PC1 subdomains (Zgurskaya *et al.*, 2008; Symmons *et al.*, 2009). If AcrA has a role in facilitating recruitment of hydrophobic substrates into this binding pocket it could then impose an element of substrate specificity which may contribute to the different susceptibility profiles of the mutants.

Inactivation of *acrA* attenuated the ability of *Salmonella* Typhimurium to invade INT-407 cells and to adhere to and invade RAW 264.7 cells *in vitro* (Figures 4.1 and 4.2). This fits well with other evidence for AcrAB-TolC in *S. Typhimurium*, and for RND systems of other species, showing that lack of pump components impairs virulence in *in vivo* infection models as well as attenuating virulence-related phenotypes such as adhesion and invasion to eukaryotic cells *in vitro*. Particularly relevant to this project is that lack of *S. Typhimurium* AcrB or TolC reduced invasion in the tissue culture model of infection and lack of TolC also reduced adhesion (Buckley *et al.*, 2006). Furthermore, *acrB* and *tolC* mutants were less able to colonise and persist in the avian gut (Buckley *et al.*, 2006) and had attenuated virulence in the mouse model causing a delay in mortality (Nishino *et al.*, 2006). However, this is the first time that the role of the PAP in this process has been determined. Furthermore, it has been shown that the AcrA mutant phenotype is similar, but distinct from the mutants lacking AcrB or TolC. This is also illustrated by recently reported transcriptomic data showing broad differences in gene expression between mutants lacking different components of AcrAB-TolC (Webber *et al.*, 2009). Only four genes were differentially expressed in a similar manner in all three mutants (Figure 6.1). The *acrA* mutant had the lowest number of genes with altered transcription, 115, while 171 genes had altered transcription in the *tolC* mutant. The strain with by far the most genes with altered expression was the *acrB* mutant (L110) with 569 genes differentially expressed. The strains lacking AcrB and TolC had the most similar transcriptomes having 133 expression changes in common. In contrast, the *acrA* mutant was the most distinct, with 24 gene expression changes in common with

Figure 6.1 Comparison of the transcriptomes of the *acrA*, *acrB* and *tolC* mutants



From Webber *et al.*, 2009

Venn diagram showing the overlap in genes with significantly altered expression in the mutants compared to SL1344. In each category, regulators with altered expression are indicated and those shown in red have increased expression while those shown in blue have decreased expression compared to SL1344.

the *acrB* mutant and none that were changed only in the *acrA* mutant and the *tolC* mutant (Figure 6.1).

The direct role of RND pumps in virulence and in virulence-related phenotypes is now well established but the reasons for this are not fully understood. Recent work in this laboratory has shown that one cause of the attenuation of the mutants is the change in expression of virulence-related genes (Webber *et al.*, 2009). Strains lacking *acrB* and *tolC* had reduced expression of genes found in SPI-1 and a consequent reduction in secretion of the SPI-1 effectors SipA, SipB and SipC. In contrast, in the *acrA* mutant, genes found in SPI-2 showed widespread decrease in expression, while SPI-1 gene expression was unaffected. Reduced expression of chemotaxis and motility genes were detected after inactivation of *acrB* or *tolC*, and in the *acrB* mutant this led to decreased motility. Expression of genes known to be involved in anaerobic respiration had decreased expression in the *acrB* mutant but increased expression in the *acrA* mutant. However, both these mutants had an impaired ability to grow anaerobically which could contribute to their reduced virulence *in vivo* (Webber *et al.*, 2009).

As already discussed, lack of components of AcrAB-TolC impair the level of efflux function in *S. Typhimurium*. Consequently, a logical second hypothesis for the attenuation of mutants lacking components of RND systems is that reduced efflux of a factor or factors renders the strains less able to cause infection. Initial evidence for this hypothesis was presented by Hirakata and colleagues (2002) who showed that the ability of a triple efflux mutant (*mexAB-oprM*) to invade MDCK cells *in vitro* could be restored by addition of supernatant collected from an infection of MDCK cells with wild-type *P. aeruginosa*. They concluded that a factor required for invasion was exported by MexAB-OprM in wild-type *P. aeruginosa* and that addition of the supernatant could restore the invasiveness of the mutant by exogenously providing this factor (Hirakata *et al.*, 2002). In contrast, the same effect was not seen in *Salmonella* when these experiments were recreated. Addition of supernatant

collected from INT-407 cells infected with SL1344 could not restore the invasiveness of the *tolC* mutant (Figures 5.2-5.6). Furthermore, co-infection of the strains lacking AcrA, AcrB or TolC with SL1344 was also unable to restore the attenuation of the mutants (Figures 5.7 and 5.8). This began to suggest that the attenuation of the *Salmonella* efflux mutants was not caused by the lack of an exported factor as neither supernatant from wild-type infection, or presence of the wild-type itself were able to restore the defect.

Two alternative interpretations of the Hirakata *et al.*, (2002) data were explored. Firstly, it was shown that media conditioned by INT-407 cells alone was unable to restore the invasiveness of the mutant so it is unlikely that a response to a eukaryotic factor in the mutant was responsible for the restoration of the mutant invasiveness. Secondly, it was postulated that the restoration in invasiveness seen in *P. aeruginosa* after addition of culture supernatant, collected from MDCK infected with the wild-type strain, was due to addition of a 'virulence related factor' which is a normal product of the wild-type bacteria, not exclusively produced during infection, but which is not produced, or not efficiently exported by the efflux mutants. Media conditioned by the growth of SL1344 was able to at least partially restore the ability of the *acrA*, *acrB* and *tolC* mutants to adhere to and invade INT-407 cells leading to the conclusion that a compound, produced or exported by SL1344 but not the mutants, is required for invasion and that exogenous addition of this factor/s can restore invasion (Figures 5.10 to 5.13). It is postulated that the same effect was not seen after addition of supernatant or after co-infection with SL1344 because the 'virulence related factor' or factors did not reach a sufficiently high concentration.

It is likely that the restorative ability of the SL1344 conditioned media is the result of multiple factors. This is because pre-boiling the SL1344 conditioned media ablated its ability to complement the invasion defect in L109 (*tolC::aph*) but did not affect the restoration of in the *acrA* and *acrB* mutants. This suggests that multiple factors, some of which are heat sensitive while some are not, are involved

in the restorative ability of SL1344 conditioned media. It is possible that additional factors are required to restore the ability of the *tolC* mutant to invade INT-407 cells as TolC functions with various other systems which may contribute to its attenuation. Further work is underway to identify the components of the SL1344 conditioned media which are able to restore the ability of the mutants to invade INT-407 cells.

One possibility is that the virulence related factor is a metabolic product or other small soluble factor. It has been suggested that TolC of *E. coli* is involved in the export of such metabolic products and that these products of normal cellular metabolism accumulate in TolC mutants as they are not exported as efficiently (Imuta *et al.*, 2008; Rosner and Martin, 2009). In addition, it has been reported that reduced export of the metabolic product formate was linked to reduced expression of SPI-1 and attenuated ability of *Salmonella* to invade HEp-2 cells *in vitro* (Huang *et al.*, 2008). These data together suggest that the active factor of the SL1344 conditioned media could be a product of normal cellular metabolism, required for invasion, which is not exported in the TolC mutant and exogenous addition of which is able to restore the ability to invade INT-407 cells. A comparison of the secreted metabolites of the TolC mutant and SL1344 could help identify such a molecule and this would greatly enhance our understanding of the role of AcrAB-TolC in virulence and virulence-related phenotypes.

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Appendix 1 Figure 1. The *acrA* gene with primer locations indicated

gccacatccaggatgtgttgcgtgtctccagcgcttggtgtttgggtttttcgtgccata
 tgtcgggtgaatttacaggcggttagatttacatacatttatggatgtatgtaccatagcac
 gacgataatataaacgcagcaatgggtttaaggaccttgaccattgaccaatttgaaat
 cggacactcgaggtttacatATGAACAAAAACAGAGGGTTAACGCCTCTGGCGGTCGTTC
 TGATGCTCTCAGGCAGCTTAGCGCTAACAGGATGTGACGACAAACAGGACCAGCAAGGCG
 GCCAGCAGATGCCAGAAGTTGGGGTTGTACACTAAAAACGGAACCACTGCAAATCACAA
 CTGAACTTCCGGGTTCGTACCGTTGCTTACCGTATCGCCGAAGTTCGCCCCGAGGTAAGCG
 GCATTATCCTGAAGCGTAATTTTCGTTGAGGGAAGTGATATCGAAGCGGGAGTCTCTCTCT
 ATCAGATTGATCCTGCGACCTACCAGGCGACTTACGACAGCGCTAAGGGCGATCTGGCAA
 AAGCGCAGGCCCGCCGCAATATCGCTGAACTGACGGTGAAGCGTTATCAAAAGCTGCTGG
 GTACGCAGTACATCAGTAAGCAGGAATACGATCAGGCGCTGGCTGACGCACAACAAGCGA
 CTGCCGCTGTTGTTCGACGCAAAAGCCGCCGTTGAAACCGCACGTATCAACCTGGCGTATA
 CCAAAGTCACCTCGCCGATTAGCGGTCGTATTGGTAAGTCATCCGTAACGGAAGGCGCAC
 TGGTACAGAACGGTCAGGCGTCGGCGCTGGCGACAGTGCAGCAGCTGGACCCTATTTATG
 TCGATGTGACCCAGTCCAGCAATGACTTCCTGCGCCTGAAGCAGGAGCTGGCAAATGGTT
 CGCTGAAACAGGAAAACGGCAAAGCGAAGGTTCGACCTGGTGACCAGCGACGGTATCAAAT
 TCCCGCAGTCCGGTACGCTTGAGTTCTCCGACGTGACCGTTGACCAAACACCGGGTCTA
 TTACTTTGCGCGCCATCTTCCCTAACCCGGATCACACCTTATTGCCAGGAATGTTTCGTTT
 GCGCACGTCTGCAGGAAGGGACAAAACCGACGGCATTACTGGTTCCACAACAGGGCGTTA
 CCCGTAATCCACGCGGATGCCACGGTGCTGGTGGTTGGCGCTGATAACAAAGTGGAAA
 CCCGCCAAATCGTCGCAAGCCAGGCGATCGGCGATAAGTGCTGGTGACTGACGGATTGA
 AAGCGGGCGACCGCGTAGTCGTACGCGGCTGTGCAAAAAGTACGTCCTGGCGCACAGGTTA
 AAGTACAGGAAATTACCGCGGATAACAAACAGCAAGCCGCAAGCGGTGATCAACCTGCTC
 AGCCCAGGTCTTAActtaaacaggagccgttaagacatgcctaatttctttatcgatcgc
 cctatatatttgcggtgggtgatcgccatcatcatcatgttggcaggggggctcgcgatcctc
 aaattgcccgttagcgcaatatccgacgattgcgccaccagcagtgacgatctccgcaacc
 taccctggcgctgatgcgaaaacggtacaggata

The *acrA* gene is shown in capital letters while the upstream and downstream flanking regions are shown in lower case. Blue highlighted regions indicate primers 568 and 569 (Table 2.4) which were used to amplify the construct containing the *aph* gene for inactivation of the gene. Red highlighted regions indicate the upstream and downstream check primers 570 and 571 (Table 2.4). Yellow highlighting indicates the start codon of *acrA* while light grey highlighting indicates the start codon of *acrB*.

Appendix 1. Figure 2. The predicted sequence *acrA* region after insertion of the *aph* gene by homologous recombination

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gccacatccaggatgtgttgcgtgtctccagcgcttgttgttttggttttttcgtgccata
tgtcgggtgaatttacaggcggttagatttacatacatttatggatgtatgtaccatagcac
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cggacactcgaggtttacatATGAACAAAAACAGAGGGTTAACGCCTCTGGCGGTCGTTC
TGATGCTCTCAGGCAGCTTAGCGCTAACAGGATGTGACGACAAACAGGACCAGCAAGGCG
TGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAG
GAACTTCAAGATCCCTCAGCTGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAA
GCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTA
CTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGG
GCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCAGGAATTGCC
AGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAACTGGATGGCTTTCTT
GCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATC
GTTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAG
GCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCG
GCTGTACAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCGGTGCCCTGAA
TGAAGTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGC
AGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCC
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TGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAA
ACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCT
GGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACGTTCGCCAGGCTCAAGGCGCGCAT
GCCCCAGCGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGT
GGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTA
TCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGA
CCGCTTCTCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGAGCGCATCGCCTTCTATCG
CCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACG
CCCAACCTGCCATCACGAGATTTTCGATTCCACCGCCGCTTCTATGAAAGGTTGGGCTTC
GGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAG
TTCTTCGCCCACCCAGCTTCAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGG
AACTTCGGAATAGGAACCTAAGGAGGATATTCATATGGACCATGGCTAATCCCGCTGCAA
AAAGTACGTCCTGGCGCACAGGTTAAAGTACAGGAAATTACCGCGGATAACAAACAGCAA
GCCGCAAGCGGTGATCAACCTGCTCAGCCCAGGTCTTAACTtaaacaggagccgttaaga
Catgcctaattttctttatcgatcgccctataatttgccgtgggtgatcgccatcatcatcat
Gttggcaggggggctcgcgatcctcaaatggccggtagcgcaatatccgacgattgcgcc
accagcagtgacgatctccgcaacctaccctggcgctgatgcgaaaacggtacaggata

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The *acrA* gene is shown in capital letters while the upstream and downstream flanking regions are shown in lower case. Blue highlighted regions indicate primers 568 and 569 (Table 2.4) which were used to amplify the construct containing the *aph* gene for inactivation of the gene. Red highlighted regions indicate the upstream and downstream check primers 570 and 571 (Table 2.4). Yellow highlighting indicates the start codon of *acrA* while light grey highlighting indicates the start codon of *acrB*. The underlined region indicates the *aph* gene and the start and stop codons are indicated in dark grey. Pink highlighted regions indicate primers 237 and 239 which are internal to the *aph* gene.

Appendix 1. Figure 3. Alignment of the expected sequence for the disrupted *acrA* region with the upstream sequencing data for L823 (*acrA::aph*)

The inactivation primers are indicated in blue. The yellow highlighting indicates the start codon of the *acrA* gene and the dark grey highlighting indicates the start codon of the *aph* gene.

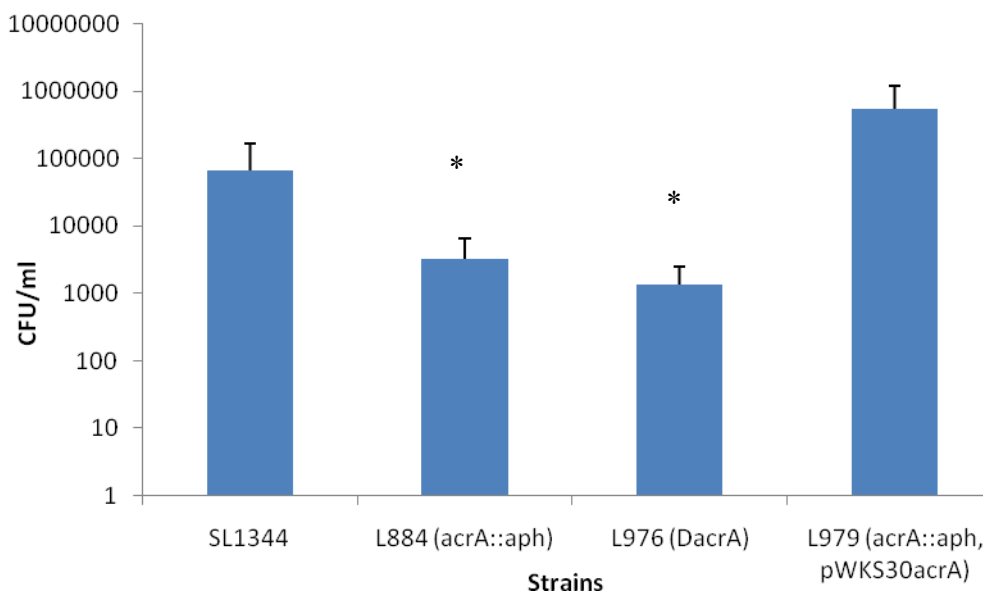
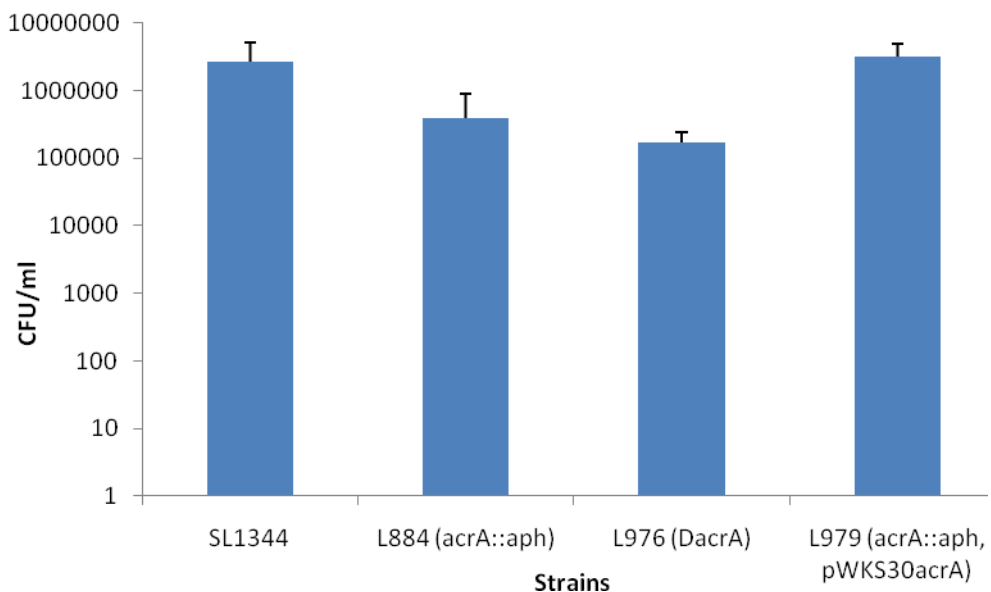
Expected L823	TGTCGGTGAATTTACAGGCGTTAGATTTACATACATTTATGGATGTATGTACCATAGCAC CGTCGGTGAATTTACAGGCGTTAGATTTACATACATTTATGGATGTATGTACCATAGCAC
Expected L823	GACGATAATATAAACGCAGCAATGGGTTTAAGGACCTTTGACCATTGACCAATTTGAAAT GACGATAATATAAACGCAGCAATGGGTTTAAGGACCTTTGACCATTGACCAATTTGAAAT
Expected L823	CGGACACTCGAGGTTTACATATGAACAAAAACAGAGGGTTAACGCCTCTGGCGGTCGTTCT CGGACACTCGAGGTTTACATATGAACAAAAACAGAGGGTTAACGCCTCTGGCGGTCGTTCT
Expected L823	TGATGCTCTCAGGCAGCTTAGCGCTAACAGGATGTGACGACAAACAGGACCAGCAAGGCG TGATGCTCTCAGGCAGCTTAGCGCTAACAGGATGTGACGACAAACAGGACCAGCAAGGCG
Expected L823	TGTAGGCTGGAGCTGCTTCTGAAGTTCTATACTTTCTAGAGAATAGGAACTTCGGAATAG TGTAGGCTGGAGCTGCTTCTGAAGTTCTATACTTTCTAGAGAATAGGAACTTCGGAATAG
Expected L823	GAACTTCAAGATCCCCTCACGCTGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAA GAACTTCAAGATCCCCTCACGCTGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAA
Expected L823	GCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTA GCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTA
Expected L823	CTGGGCTATCTGGACAAGGGAAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAAGTGG CTGGGCTATCTGGACAAGGGAAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAAGTGG
Expected L823	GCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCAGGAATTGCC GCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAAGCGAACCAGGAATTGCC
Expected L823	AGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTT AGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTT
Expected L823	GCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATC GCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATC
Expected L823	GTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAG GTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAG
Expected L823	GCTATTTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCG GCTATTTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCG
Expected L823	GCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAA GCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGANCGACCTGTCCGGTGCCCTGAA

Appendix 1. Figure 4. Alignment of the expected sequence for the disrupted *acrA* region with the downstream sequencing data for L823 (*acrA::aph*)

The inactivation primers are indicated in blue. The yellow highlighting indicates the stop codon of the *acrA* gene and the dark grey highlighting indicates the stop codon of the *aph* gene.

Expected L823	GCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTTCGACCACCAAGCGAAA GCAATGCGGCGN-TGCATACGCTTGATCCGGCTACCTGCCCATTTCGACCACCAAGCGAAA
Expected L823	CATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTG CATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTG
Expected L823	GACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTGTTCGCCAGGCTCAAGGCGCGCATG GACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTGTTCGCCAGGCTCAAGGCGCGCATG
Expected L823	CCCACGCGGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTG CCCACGCGGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTG
Expected L823	GAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTAT GAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTAT
Expected L823	CAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGAC CAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGAC
Expected L823	CGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGAGCGCATCGCCTTCTATCGC CGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGAGCGCATCGCCTTCTATCGC
Expected L823	CTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGC CTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGC
Expected L823	CCAACCTGCCATCACGAGATTTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCG CCAACCTGCCATCACGAGATTTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCG
Expected L823	GAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGT GAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGT
Expected L823	TCTTCGCCCACCCAGCTTCAAAAGCGCTCTGAAGTTCTATACTTTCTAGAGAATAGGA TCTTCGCCCACCCAGCTTCAAAAGCGCTCTGAAGTTCTATACTTTCTAGAGAATAGGA
Expected L823	ACTTCGGAATAGGAACCTAAGGAGGATATTCATATGGACCATGGCTAATCCCGCTGCAAA ACTTCGGAATAGGAACCTAAGGAGGATATTCATATGGACCATGGCTAATCC-GCTGCAAA
Expected L823	AAGTACGTCCTGGCGCACAGGTTAAAGTACAGGAAATTACCGCGGATAACAAACAGCAAG AAGTACGTCCTGGCGCACAGGTTAAAGTACAGGAAATTACCGCGGATAACAAACAGCAAG
Expected L823	CCGCAAGCGGTGATCAACCTGCTCAGCCCAGGTCTTAACCTTAAACAGGAGCCGTTAAGAC CCGCAAGCGGTGATCAACCTGCTCAGCCCAGGTCTTAACCTTAAACAGGAGCCGTTAAGAC

Appendix 2. The ability of *S. Typhimurium* SL1344 and various efflux mutants thereof to adhere to and invade mouse macrophages (RAW264.7)



Experiments performed by Sarah Coleman, a third year undergraduate project student. Data are displayed as mean of at least three separate experiments performed in triplicate + standard deviation. Values returning a p value of ≤ 0.05 from a Student's T-test comparing mutants to the SL1344 values are denoted by *.

Publications resulting from this study

1. Publications

Jessica M A Blair, Roberto M La Ragione, Martin J Woodward and Laura J V Piddock

The Periplasmic Adaptor Protein AcrA has a Distinct role in Antibiotic Resistance and Virulence of *Salmonella enterica* Serovar Typhimurium.

Journal of Antimicrobial Chemotherapy, In press.

Mark A. Webber, Andrew M. Bailey, **Jessica M. A. Blair**, Eirwen Morgan, Mark P. Stevens, Jay C. D. Hinton, Al Ivens, John Wain, and Laura J. V. Piddock

The Global Consequence of Disruption of the AcrAB-TolC Efflux Pump in *Salmonella enterica* Includes Reduced Expression of SPI-1 and Other Attributes Required To Infect the Host.

Journal of Bacteriology, July 2009, p. 4276-4285, Vol. 191, No. 13

Jessica M.A. Blair and Laura JV Piddock.

Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Current Opinion in Microbiology, In press

2. Poster presentations at international conferences

Jessica M A Blair, Roberto M La Ragione, Martin J Woodward and Laura J V Piddock

The role of AcrA in Antibiotic Resistance and Virulence of *Salmonella enterica* Serovar Typhimurium.

American Society for Microbiology, 108th General Meeting, 2008. Poster number A-094

Jessica M A Blair, Roberto M La Ragione, Martin J Woodward and Laura J V Piddock

The Periplasmic Adaptor Protein AcrA has a Distinct role in Antibiotic Resistance and Virulence of *Salmonella enterica* Serovar Typhimurium.

Gordon research conference on Multi-drug efflux systems, Galveston, 2009.

3. Poster presentations at national conferences

Jessica M A Blair, Roberto M La Ragione, Martin J Woodward and Laura J V Piddock

The role of AcrA in Antibiotic Resistance and Virulence of *Salmonella enterica* Serovar Typhimurium.

Society for General Microbiology, March-April 2008.